Phytophthorols–Novel Lipids Produced by *Phytophthora cactorum*

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Lipid-phytophthorols are a series of saturated and unsaturated odd chain fatty alcohols (C_{17}-C_{21}) esterified to myristic acid (14:0) with each possessing two hydroxyl functions in a 1,2-diol relationship centered toward the ester terminus. They have been identified, and their biosynthetic relationship to other fatty acid and alcohols produced by *Phytophthora cactorum* have been determined. Because phytophthorols are crystalline materials and elute in TLC and HPLC with sterols, an architectural function is implied. In the absence of cholesterol, dietary phytophthorol failed to induce reproduction.
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There are cogent reasons to believe that fatty acids play multiple roles: structural and reserve energy components of mycelia and spore, and in developmental regulation of the Oomycetes life cycle (1–3). However, the physiological importance of accumulating select acyl lipids, or producing additional end products derived from the pathway, e.g. very-long chain fatty alcohols (VLFA) and prostaglandins, is not clear. We have considered the possibility that the genes that control fatty acids, alcohol biosynthesis and metabolism are distributed generally throughout the oomycetous fungi. However, their expression is developmentally and compartmentally regulated so that the fitness (structure and amount) for one or more acids and alcohols to act functionally is temporally coordinated and selected by the different taxa as a result of Darwinian evolution. In order to pass through one life cycle or switch from one developmental stage into another, the organism must utilize specific fatty acids and alcohols with each one (or groups) occurring at some threshhold level in an appropriate architectural fraction and at a proper time in relation to other compounds, e.g. sterols.

In our studies with the Oomycetes, we earlier observed that the mycelia of *Phytophthora cactorum* produced a range of fatty acids and alcohols (4–6). The utility of some of these lipids in mediating growth and reproduction is presented in the present communication. Previously, [2-^{14}C]acetate supplied to *P. cactorum* was shown to be incorporated into acyl polar lipids, i.e. phospholipids and sphingolipids, triglycerides, VLFA and what appeared to be a steroidal compound based on its chromatographic behavior (4–6). However, we also had shown that the fungus lacked a completed sterol pathway (7,8). The results reported here describe the identification, biosynthesis and possible function of this unknown compound(s), which is(are) shown to be lipid but nonsteroidal. The physiological significance and biosynthetic interrelationships of the unknown lipids with other lipids produced by Oomycetes also is discussed.

MATERIALS AND METHODS

Organism. P. cactorum (University of California, Berkeley strain 51–22) was grown on a completely defined media (9). Growth was initiated by seeding the flasks with mycelia previously cultured on lipid-free media. For studies on reproduction, agar was added to solidify the media (10). Agar, which is derived from algae, retains after industrial processing, sterol (7) and unsaturated fatty acids (Nes, unpublished). The cultures routinely were monitored visually for fungal and bacterial contamination. Had a fungal spore been introduced into the cultures, ergosterol would have been detected; none was observed.

Chemicals. [1-^{14}C]Palmitic acid (55 mCi/mMol) was purchased from Amersham, Searle, Arlington Heights, IL. Methyl ester fatty acid standards were purchased from Supelco, Bellefonte, PA. Sterol and VLFA standards previously were prepared in this laboratory (11). Solvents were either redistilled reagent-grade or high-performance liquid chromatography-(HPLC) grade.

Lipid extraction and analysis. Mycelia were cultured at 20 C in the dark in 250 ml flasks supplemented with 50 ml media for two to three wk without shaking as previously described (10). For reproductive studies, agar-solidified media were dispensed into petri dishes (7). The wet mycelia were harvested and then ground with sea sand and acetone, and the material was extracted in a Soxhlet apparatus overnight with refluxing acetone. The acetone extract was applied as a band onto a thin layer chromatography (TLC) plate (developed in benzene-ether, 9:1) and bands matching the origin (polar lipids: phospholipids, glycosides and free acids), ester or front region, and R_f: 0.3 to 0.4 (cholesterol standard), 0.4 to 0.45 (lophenol standard) and 0.48 to 0.56 (lanosterol and triacontanol standards) were eluted from the plate with a series of solvents-chloroform/methanol, hexane, ether and benzene. The TLC bands were made visible by spraying the plates with an aqueous solution of H_2SO_4 followed by charring. When radioactive material was used in the incubations, the eluted zones were transferred to scintillation vials, and the solvent was removed by a stream of nitrogen. Diphenyloxanzole-benzene (POPOP) cocktail (5 ml) was added to the vial and the radioactivity determined using a scintillation counter at 90% efficiency for ^{14}C (11). The media was extracted three times with diethyl ether; the combined ether extracts were analyzed immediately by TLC and gas liquid chromatography (GLC).

To determine the fatty acid content, each of the materials eluted from the five TLC zones was reacted with BF_3 in methanol and the resultant methyl esters chromatographed on packed (Hi-Eff and carbowax columns) and

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Abbreviations: AA, [1-^{14}C]arachidonic acid; CI, chemical ionization; EI-MS, electron impact-mass spectra; EPA, eicosapentaenoic acid; FT, Fourier transform; GC-MS, gas chromatography-mass spectrometry; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; LAH, lithium aluminum hydride; OH, hydroxyl; POPOP, diphenyloxanzole-benzene; RP-HPLC, reversed-phase-high performance liquid chromatography; TLC, thin layer chromatography; VLFA, very long chain fatty alcohols.

capillary (DB5-30 m) columns. The elution times of the samples on the various columns were compared with the times of transmethylated fatty acid standards. Molecular weights were verified by gas chromatography-mass spectrometry (GC-MS). Packed columns were operated isothermally at 180 C; for the capillary column, a temperature program run was employed; column temperature was programmed at a linear rate of 5 C/min from 175–225. The configuration of the double bands of oleic acid, 18:1 (Δ^9-*cis*); linolenic acid, 18:2 ($\Delta^{9,12}$*cis,cis*) α-vs-γ-linoleic acid 18:3 ($\Delta^{9,12,15}$ vs $\Delta^{6,9,12}$); and arachidonic acid, 20:4 ($\Delta^{5,8,11,14}$ all *cis*) were determined by co-chromatography of the methyl esters on $AgNO_3$ TLC plates with authentic standards (10% Ag^{++} impregnated into silica developed with hexane-benzene or chloroform) and then by GLC of the eluted bands using carbowax and capillary columns.

The TLC bands at R_f–0.3 to 0.5 also were examined by GLC without saponification, using 3% SE-30 and 3% OV-17 packed columns in which the columns were operated isothermally or in the same temperature program mode (240 to 280 C at 0.5 C/min); retention times (at 245 C) were relative to cholesterol, RRT_c.

GC-MS and direct probe-MS were carried out with a VG-micromass 70/70-HS mass spectrometer equipped with a Hewlett Packard 5880 GC. Electron ionization (EI) spectra were determined at 70 electron volts with the ion source temperature at 150 C. Chemical ionization spectra were obtained using isobutane. A Nicolet 200 operating in the Fourier transform (FT) mode at 200 MHz was used to obtain the ^{1}HNMR spectra of samples dissolved in deuterated chloroform. Acetate (acetic anhydride/pyridine or via diazomethane) and trimethyl silyl ether (Me_3Si) derivatives were prepared by standard methods. Reversed-phase HPLC was performed using a C_{18} column eluted with 4% aqueous methanol. The pump speed was set at 1.6 ml/min and the detector set at 205 nm. Fractions were collected every min, cholesterol elutes in 24 min and docosanol (C_{22}) elutes in 19 min. Sitosterol and higher long-chain fatty alcohol homologues (C_{24} and C_{26}) eluted in the 26 and 40 min fractions.

RESULTS

Phytophthorol identification. The mycelial acetone extract of *P. cactorum* yielded five major TLC zones at R_f: 0.00, 0.35, 0.41, 0.53 and 0.96. When the acetone extract was hydrolyzed with 10% methanolic KOH the TLC zones at R_f 0.35 and 0.41 disappeared. This immediately suggested that the material at R_f 0.35 and 0.41 was neither a triterpenoid nor a steroid. The combined materials at R_f 0.35–0.43 were crystalline rather than oily and possessed a broad melting point ca. 75–80 C. The unsaponified materials that migrated at R_f 0.43 were examined further by GLC. Two major peaks were evident in a chromatogram that ran substantially after sterols and triterpenoids, viz. RRT_c – 3.77 and 6.20 on OV-17 packed columns operated at 245 C. In the temperature program mode, multiple peaks were detected (Fig. 1). The GLC peaks appeared successively by C_2H_4 homologues as indicated by comparison of semilogarithmic plots constructed on the unknowns profile (C-length vs retention time) with similar plots constructed using VLFA. GC-MS of the three major peaks (ms obtained at center of peak) produced spectra with M^+ – 494, 522 and 550, respectively. High-resolution mass measurements of the three compounds indicated formulas of $C_{31}H_{60}O_4$, $C_{33}H_{64}O_4$ and $C_{35}H_{68}O_4$, respectively. Hydrolysis of the unknowns followed by acidification (6 N HCl) of the aqueous extract and then esterification (BF_3-methanol) of the heptane extractable material produced a single compound that co-chromatographed with myristic acid (14:0) in GLC. At this point, the unknowns at R_f 0.35 and 0.46 were believed to be a series of fatty acid derived compounds that possessed an acid and alcohol portion with an ester linkage. Since we found only a single fatty acid, the homologation presumedly was due to changes in the alcohol rather than the acid portion. We assumed the polar TLC-mobility was due to OH-groupings in the alcohol portion. If it was an ester compound, the material would have run to the front of the TLC.

In an earlier study, we observed that radioactivity from a [2-^{14}C]acetate feed to *P. cactorum* was incorporated into unknown materials that co-chromatographed with sterols in TLC (4) and reversed-phase high-performance liquid chromatography (RP-HPLC) (Nes, unpublished). When the material, which chromatographed at R_f–0.4, was injected into the HPLC and the fractions collected where sterols normally elute (24 to 28 min), only one principal homologue was detected by GLC (M^+-494). Its electron impact-mass spectra (EI-MS) is shown in Figure 2. The GC-MS of this material using the capillary column indicated a minor compound eluting in the tail with M^+-492. This compound was not evident readily, using the packed columns in GC-MS (EI-MS of the Me_3Si derivative of the minor compound is shown in Figure 3). ^{1}HNMR, with assignment of the first major Phytophthorol eluting in GLC) now referred to as Phytophthorol-1; compound having M^+-494), exhibited chemical shifts at 0.87 (t,J = 6.0 Hz, terminal methyl on an aliphatic chain), 1.24 (S, internal aliphatic chain of CH_2), 2.36 (t,J = 6.5 Hz, CH_2 alpha to a carbonyl group), 4.14 (m,J = 9 Hz, CH_2 alpha to oxygen, thereby implicating an ester function), 5.09

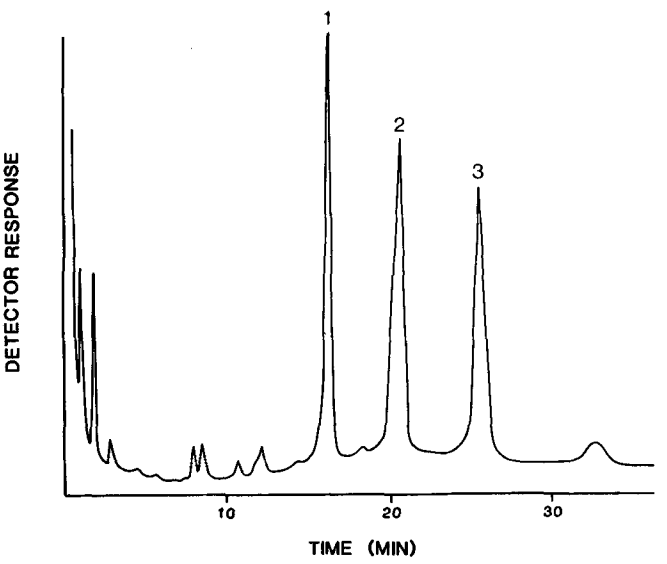

FIG. 1. GLC (packed 3% SE-30 column operated in the temperature program mode) of a mixture of phytophthorols isolated from the mycelia of *P. cactorum*.

and 5.36 (m,J = 3 Hz each, two hydroxyl groups in a 1,2-relationship) ppm. Although this phytophthorol failed to be acetylated by the acetic anhydride in pyridine method, it was acetylated with diazomethane. The spectra of the acetate showed the inclusion of an acetoxy group (2.04 ppm) and loss of the OH group by the absence of the chemical shift at 5.09 ppm. A TMS-derivative also was obtained, and the mass spectra supported the molecule having two acetylable hydroxyl groups (m^+-638). ^{1}HNMR of the Me_3Si-derivative—0.87

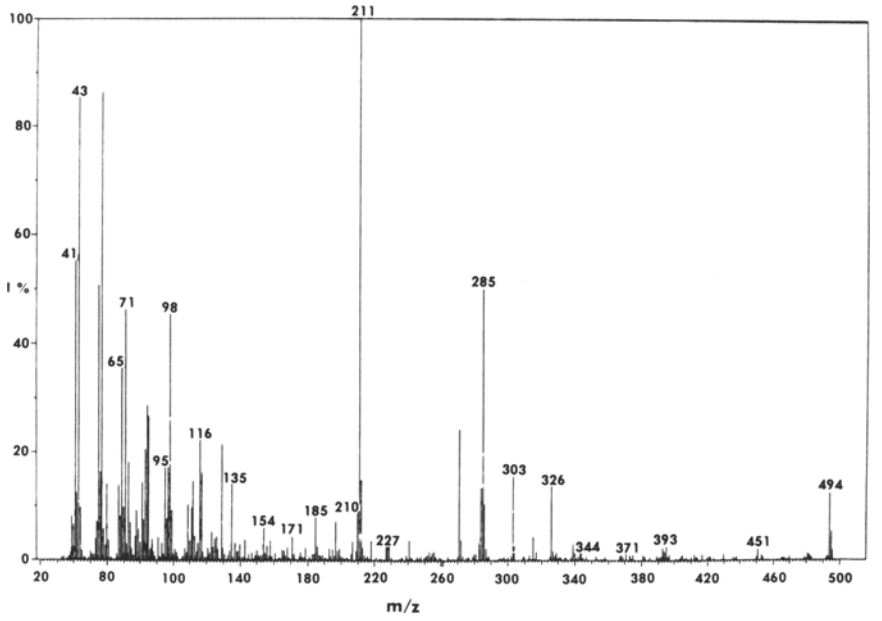

FIG. 2. EI-MS of the underivatized phytophthorol-1.

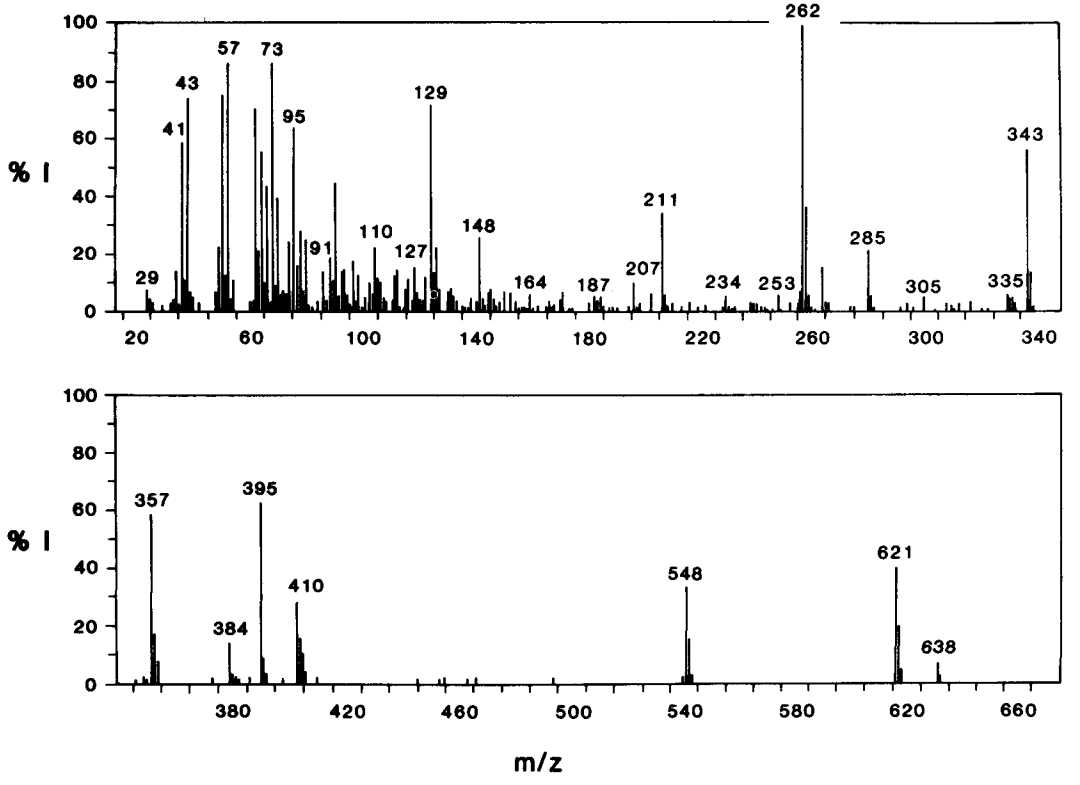

FIG. 3. EI-MS of one of the trimethylsylether derivatives of the phytophthorol series of homologues (phytophthorol-4) which possess unsaturation in the alcohol portion.

(t,J = 6 Hz), 1.24 (S), 2.43 (t,J = 4 Hz) and 4.76 (S). The peak at 4.76 ppm integrated to two protons, while the multiplet present at 4.15 ppm in the spectra of the free alcohol disappeared. These facts indicate the 2-OH groups are located close to the ester terminus. With only this information, the exact location and geometry of the two hydroxyl groups in the alcohol portion still was equivocal. The EI-MS of Phytophthorol-1 lacked any diagonistic fragments for the OH-groups. When Phytophthorol-1 was reduced with lithium aluminum hydride (LAH) and then chromatographed on reversed-phase TLC developed with methanol/H_2O (19:1), the alcohol portion (triol) migrated to R_f − 0.3. ^{1}HNMR of the sample showed a full proton (multiplet centered at 0.365 ppm, which represented the terminal OH). This material then was acetylated with diazomethane and a CI-probe obtained on the resultant derivative. Prominent fragments at m/z 403 (42%), 343 (5%), 283 (20%), 257 (20%), 227 (5%), 211 (2%), 199 (23%) and 171 (80%) characterized the hydrocarbon chain having three OH-groups. With the additional knowledge of the CI-probe data, the fragmentation pattern for the EI-MS of Phytophthorol-1 was assigned as shown in Figure 4. Phytophthorol-2 and 3 represent peaks 2 and 3 in the GLC (Fig. 1), which may be considered to be higher homologues based on the mass spectral data. Phytophthorol-4(C_{31}), 5(C_{33}) and 6(C_{35}) appear in the GLC tails of peaks 1, 2 and 3; they may be considered the unsaturated phytophthorols with the double bond located (site unknown) in the alcohol portion. Had it been in the acid portion of the esterified molecule, myristoleic acid (14:1Δ^9 *cis*) or some other suitable unsaturated fatty acid should have been detected by GLC of the transmethylated acid portion. The chromatographic and spectral data unambiquously show that the unknowns at R_f 0.46 are a homologous series of saturated and unsaturated odd-chain fatty alcohols (C_{17}-C_{21}) esterified to myristic acid (14:0), each possessing two hydroxy functions in a 1,2-diol relationship centered towards the ester terminals. These compounds (phytophthorols) are not waxy substances, and they lack interesterification common to estolide formation; therefore, a mycelial surface wax function seems unlikely. The unknowns at R_f 0.35 were found to be more highly hydroxylated phytophthorols; the location of the OH-groups currently are under investigation.

Biosynthesis of phytophthorol. When an aqueous suspension of 1 × 10^8 cpm [1-^{14}C]palmitic acid was distributed equally into two culture flasks, the growth of 18-day cultures was inhibited significantly (ca. 50% of the control cultures just entering into growth arrest). The acetone extract of the mycelia produced a radioactive sample having a specific activity per flask, 18 mg/2 × 10^7 cpm. The acetone extract was spread onto TLC plates and scanned for radioactivity as shown in Figure 5. The lack of an active incorporation of exogenous palmitate into the acetone extract and acyl lipids was not unexpected since the fungus synthesized this lipid as an end product. Similarly, in the sterol field we (12) and others (13,14) have shown dietary ergosterol (sterol end product) was not accumulated actively by Ascomycetes unless the more-advanced fungi were induced to become sterol auxotrophs. As shown in Figure 5, the [1-^{14}C]palmitate incubation produced only four radioactive zones: R_f 0.0, 0.35, 0.46 and 0.96, which sharply contrasted with the [2-^{14}C]acetate incubation to the fungus (4). The triacontanol band at R_f − 0.5, which in the earlier study was heavily labeled from [2-^{14}C]acetate, failed to incorporate

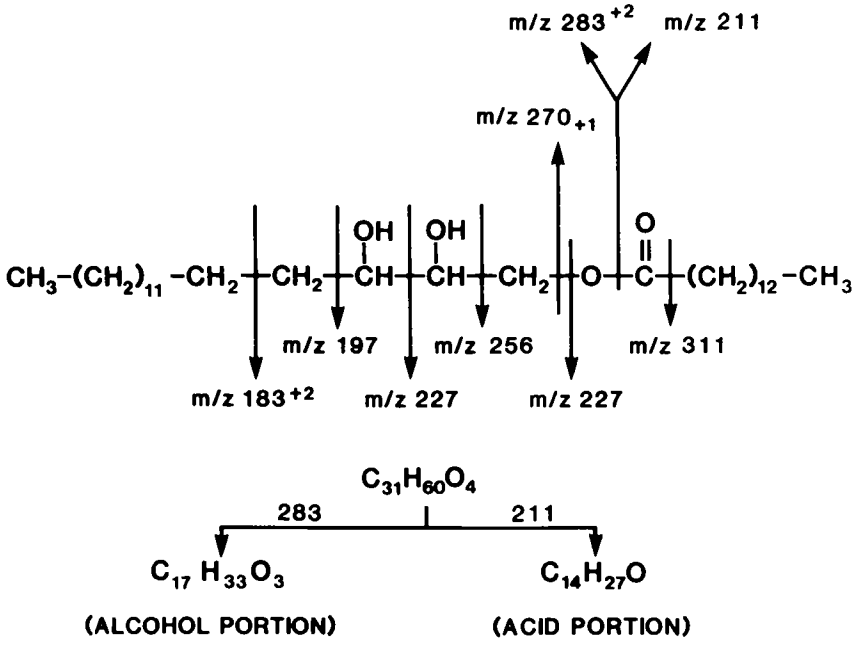

FIG. 4. Structure and mass spectral fragmentation assignment of phytophthorol-1.

detectable levels of ^{14}C from [1-^{14}C]palmitate. In order to be sure that a lower homologue in the VLFA series had not been radiolabeled (11), the material at R_f 0.4 was eluted from the plate and an aliquot reduced with LAH. Approximately 95% of the radioactivity remained at the origin when the reduced sample was chromatographed on TLC developed with benzene-ether. Both the myristic acid and alcohol fragments were converted to the monoalcohol by LAH reduction.

Since the acid fragment, now as the alcohol, moved off the origin to R_f − 0.3, leaving the triol alcohol fragment of phytophthorol at the origin, the radioactivity must be associated with the alcohol rather than the acid fragment. The material at the origin was eluted from the plate and converted to the TMS derivative. This derivatized sample together with all the radioactivity moved to the front of the TLC plate. ^{1}HNMR and GC-MS verified the chemical purity of the TLC sample as a mixture of the fatty alcohol homologues C_{17}-C_{21}. Another aliquot of the starting material at R_f 0.4 was injected into the HPLC; fractions were collected every minute for 40 min. No radioactivity appeared in the fraction corresponding to docosanol (a 19 min fraction). However, significant radioactivity appeared in the 24–28 min fractions corresponding to phytophthorol-1, followed by a few fractions in which the level of radioactivity was background. Radioactivity then appeared in subsequent fractions corresponding to the higher homologues. We have observed that docosanol can migrate into the R_f 0.4 zone under certain conditions (11). The absence of ^{14}C in the HPLC fractions corresponding to docosanol and the absence of radioactivity in the TLC-R_f 0.5 clearly show that [1-^{14}C]palmitate acid did not enter into the compartment for VLFA biosynthesis. These results differ from our other findings in which [^{14}C]palmitic acid was incorporated into VLFA produced by insects (15) and its conversion to VLFA by cell-free preparations of vascular plants (16,17).

Since [1-^{14}C]palmitic acid was incorporated into the C_{17}-alcohol, it must have been chain-elongated and then decarboxylated rather than decarboxylated before elongation. Otherwise, the radioactivity would have been lost; it was not. The media was extracted with diethylether, and the total transmethylated fatty acid composition and radioactivity was determined by GLC and TLC-radio scanning (Fig. 5). The fatty acids in the media were primarily C_{16}- and C_{18}-acids with zero and one double bond. The radioactive peak at the solvent front of the TLC plate presumably was a mixture of free acid and perhaps a trace as acyl polar lipids and prostaglandin-like materials. No labeled phytophthorols remained intact in the media, even if they were expelled with other acyl ester derivatives. A proposed biosynthetic scheme for phytophthorol-1 production from the dietary palmitic acid is shown in Figure 6.

Fatty acid esters produced by Phytophthora. Phytophthorols represent one form of fatty acid esters; other forms include acyl polar lipids and glycerides. Without sterol supplementation, logarithmically growing cultures produce ca. 0.1% dry wt phytophthorols. The total fatty acid content was approximately two orders of magnitude greater than the phytophthorol content, which was ca. 7% dry wt. The polar acyl lipids-phospholipids and sphingolipids (Nes, unpublished and [5]) and triglyceride content changed dramatically during the culture period; the fatty acids composition also changed. Triglycerides may have contained as much as 50–90% of the total esterified fatty acids, sphingolipids contained a predominance of 20:4 acid; triglycerides contain the range of acids and phytophthorols possessed a single acid, 14:0. When sterols were added to the fungus, steryl esters

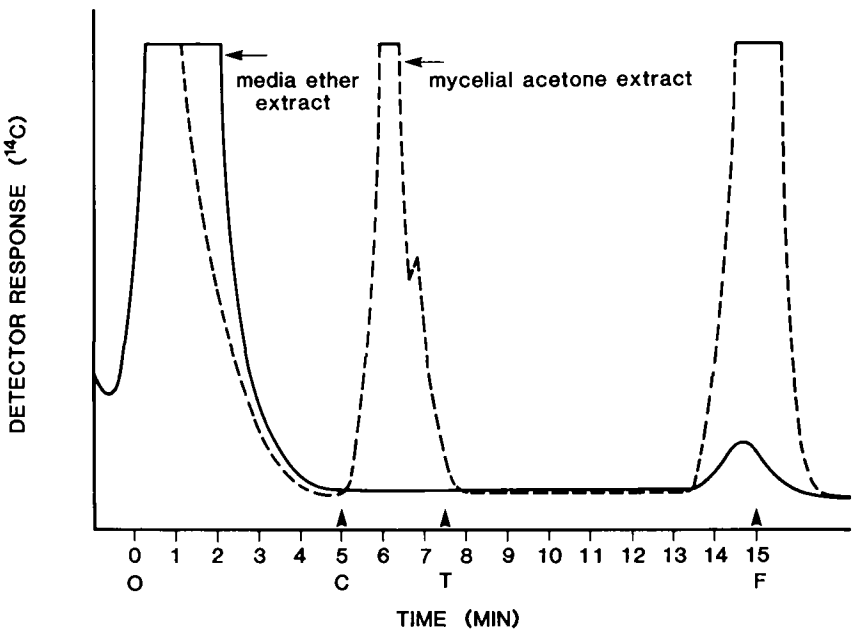

FIG. 5. TLC-radioscan of the mycelial acetone and media extracts of *P. cactorum* incubated for 18 days with [1-^{14}C]palmitate: O, origin; C, cholesterol band; T, triacontanol and lanosterol bands; F, front. TLC solvent system was benzene-ether, 9:1. Plates moved at 1 cm per min.

and acylated steryl glycosides were formed (18). The fatty acids esterified to these molecules were primarily C_{14} to C_{18} in length (19,20). In two studies on a related species of *Phytophthora* (infestans), high levels of C_{20} unsaturated acids were found to be associated with a TLC zone corresponding to steryl esters (21,22). Since the fungus was cultured under conditions in which sterol supplementation was not necessary, it was not clear whether sterol had been added to the media. If sterol had not been added, the identity of the sterol portion of the fatty acid of the sterol ester in the latter investigations would be suspect. The total fatty acid content of *P. cactorum* mycelia cultured on a completely defined media was as follows: 12:0 (tr), 14:0 (13%), 16:0 (6%), 18:0 (tr), 16:1 (tr), 18:1 (21%), 18:2 (18%), 18:3 6,9,12 (7%), 20:1 (1%), 20:2 (5%), 20:3 (10%), 20:4 5,8,11,14 (17%) and unknown (1%). When the fungus was cultured on an agar media supplemented with 10 ppm cholesterol, additional C_{20} unsaturated acids appeared in significant quantities, e.g. eicosapentaenoic acid, 20:5 $\Delta^{5,8,11,14,17}$.

Physiology of fatty acid compounds. When 1, 10, 100 or 1,000 ppm of triacontanol (C_{30}) phytophthorol-1, palmitic acid or oleic acid was incubated with *P. cactorum* in liquid or agar, solidified media growth was inhibited and reproduction was not turned on. Addition of Tween 80 and soybean lecithin (Sigma Chemical Co., St. Louis, MO) stimulated growth in a manner analogous to sterols (data not shown) and induced variable amounts of oospores, especially soybean lecithin added at high concentrations when the fungus was cultured on the Ko-media (23).

Trace levels of sterol (0.5 ppm) were sufficient to spark growth (24). This level also induced oosporogenesis. However, the number of spores produced was proportional to the dietary sterol concentrations (0.5 to 10 ppm). Thus, the stimulatory growth response was due to the sterol, not the fatty acid, supplement. Alternatively, the positive effect of certain fatty acids on reproduction may be due to the combination of having both a sterol contaminant and an unsaturated fatty acid that the fungus may require but fails to produce (cf. Discussion). When the soybean lecithin was passed through an alumina column eluted with a graded series of hexane in ether followed by chloroform/methanol (1:1) and chloroform to separate the contaminant sterol from the phospholipids, the stimulatory effect of the column purified phospholipid on growth and reproduction was substantially diminished (100% and 90%, respectively). Thus, the phospholipid alone was not responsible for growth stimulation as previously observed (25–28) or as Ko has stated for reproduction (23,29).

DISCUSSION

The total fatty acid and alcohol composition of *P. cactorum* has been elucidated in this and our earlier studies (4–6). While we have not been concerned until recently with fatty acid metabolism to prostaglandins (30–33), all Oomycetes may perform this metabolism in order to reproduce. A proposal for the biosynthetic and functional relationships of lipids produced by *P. cactorum* is shown in Figure 7. The origin of all the lipids but eicosapentaenoic acid (EPA) has been demonstrated in *P. cactorum* by incubation with appropriate labeled precursors, e.g. [2-^{14}C]acetate, [1-^{14}C]palmitate and [1-^{14}C]stearate (5 and Nes, unpublished). There are reports that [1-^{14}C]arachidonic acid (AA) is metabolized to EPA by *Achlya* (34) and *Saprolegnia* (35). However, in neither study was the starting material nor its biochemical metabolite radiochemically purified. In fact in one study (34), the AA, which

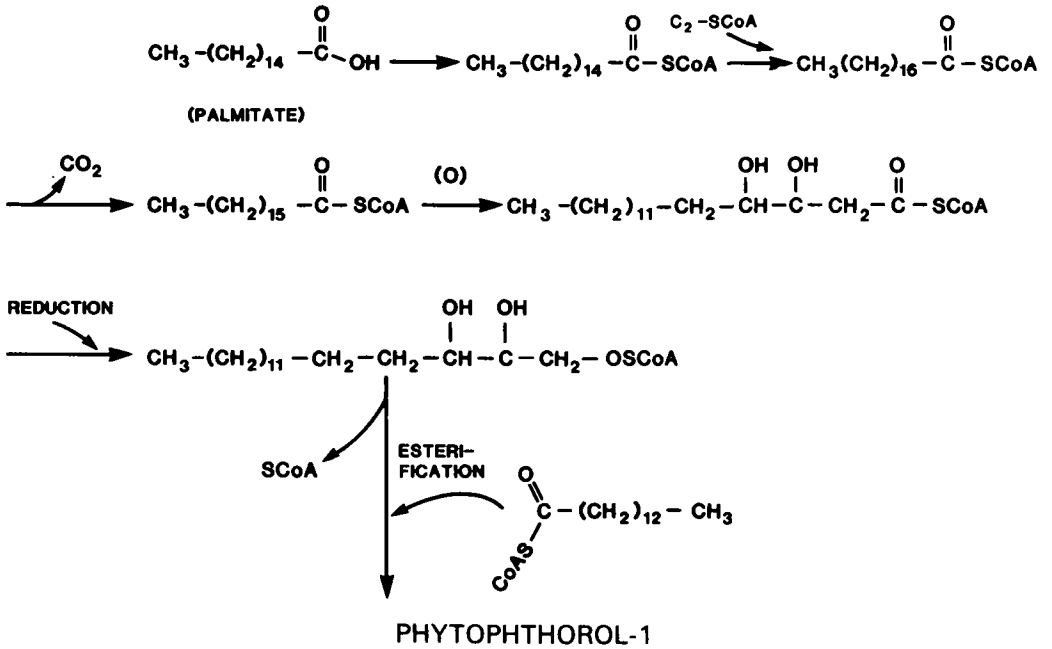

FIG. 6. Proposed biosynthetic scheme of phytophthorol-1 formation from palmitate.

was produced by biochemical rather than chemical methods, contained 4% of the 20:4 (n-3) acid. Thus, the preferential formation of EPA from 20:4(n-3) could not be ruled out. These same authors also claimed the 20:4(n-3 or $\Delta^{8,11,14,17}$) was formed following incubation with labeled stearate (18:0). The concurrent biosynthesis of C-20 isomeric unsaturated fatty acids 20:4 $\Delta^{5,8,11,14}$ and 20:4 $\Delta^{8,11,14,17}$ was difficult to accept on biosynthetic grounds (cf. Fig. 5), unless 20:3 $\Delta^{8,11,14}$ is the substrate for 20:4 $\Delta^{8,11,14,17}$. But if $\Delta^{8,11,14}$ is transformed into $\Delta^{8,11,14,17}$, why prefer using arachidonic acid as the precursor to EPA?

In a search for prostaglandins in Oomycetes, it was observed that AA was converted to metabolites of AA but not EPA (32,33). Since the desaturase cleft is assumed to fail to perform a Δ^{17}-desaturation for mechanistic reasons (36), the likelihood of a precursor-product relationship of AA with EPA seems remote. Of course, EPA could be formed by Δ^{15}-desaturation of linoleic acid; but, then a Δ^{15}-desaturase would be implicated for enzyme activity. Interestingly, Kerwin has observed the presence of α-linolenic acid and the absence of γ-linolenic acid in *Lagenidium giganteum* (a sterol-less Oomycetes [8]) in mycelia cultured with dietary phospholipids and triglycerides (38). This implies that the fungus either fails to produce γ-linolenic acid or the addition of dietary α-linolenic acid feeds back on the Δ^6-desaturase activity following its accumulation by the mycelia. To date, the cooccurrence of a Δ^6 and Δ^{15}-desaturase in nature has not been shown, although the more advanced fungi can desaturate linoleic acid to α-linolenic acid ($\Delta^{9,12,15}$) (3). If the Δ^6 and the Δ^{15}-desaturase enzymes are genetically predisposed, the oomycetous fungi temporarily may regulate the gene for Δ^{15}-desaturation until the onset of reproduction, when Δ^6-desaturase is repressed. The delayed expression of certain sterolic enzymes has been observed in fungi (12) and vascular plants (37). Another possibility is that Oomycetes do not synthesize EPA de novo and, in analogy to mammals, possess an essential dietary requirement for the acid for hormonal purposes, e.g. reproduction.

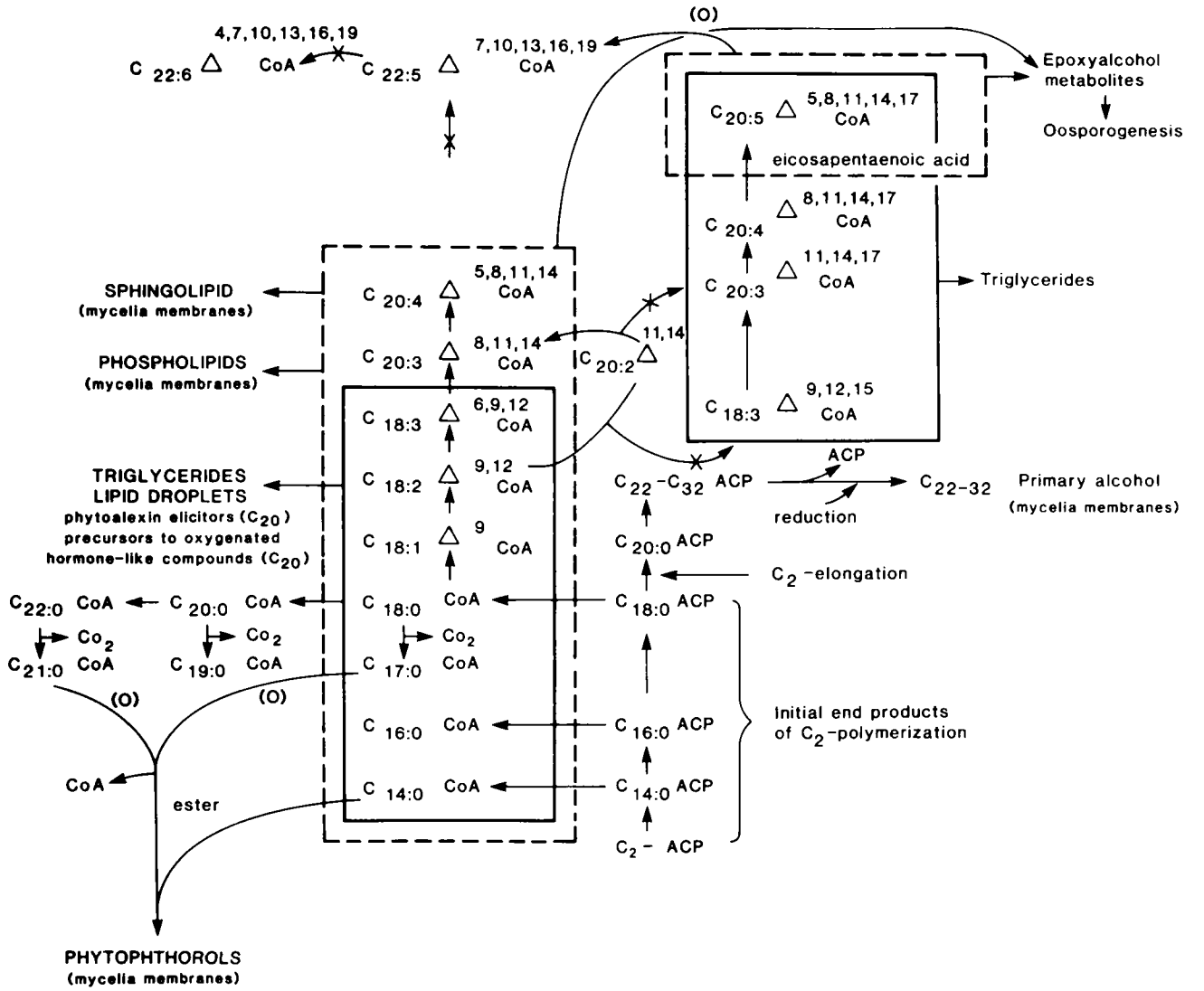

FIG. 7. Proposed biosynthetic relationships and functions of lipids produced by *P. cactorum*. X represents proposed blocks in fatty acid pathway.

The ability of Ko to induce reproduction with commercial soybean and egg lecithins in *Phytophthora* is relevant to the proposed essential fatty acid requirement by the Oomycetes (23,29). In order to stimulate oospore formation, Ko added the commercial phospholipids at 1 g/1:a; two orders of magnitude lower level, 10 mg/l (10 ppm), failed to induce oosporogenesis. It should be remembered that 10 ppm sterol induces maximal sexual sporulation (1). However, as has been shown, *Phytophthora* has the biosynthetic capacity to synthesize the head groups and dominant fatty acids that are present in the dietary phospholipid preparations. Clearly, the commercial phospholipids are not essential dietary requirements for reproduction, since these same lipids are produced by *Phytophthora*. It may be that a trace contaminant of an EPA (or other prostaglandin) precursor, e.g. α-linoleic acid, together with a contaminant sterol is present in the commercial samples, which include agar. The results of this study imply that oosporogenesis may require a dual lipid nutritional supplement: one for a select sterol and another for a select unsaturated fatty acid. If either of the two lipids are provided alone, growth, not reproduction, is affected. If the two lipids are provided together, reproduction is turned on; the number of spores produced will depend on their respective levels in the media. Presumably, the phytophthorols and VLFA replace sterols in mycelial membranes during vegetative growth. VLFA, but not phytophthorols, are present in the sterol-producing Oomycetes, *Saprolegnia ferax* (Nes, unpublished). Addition of sterols fails to change the total fatty acid composition of *P. cactorum*, although following cholesterol-induced sparking of growth the amount of fatty acid increases, and individual fatty acids associated with key ester linkages change dramatically (Nes, unpublished). This coupled with our observation that VLFA biosynthesis is compartmentalized from the fatty acid pathway leading to neutral and polar acyl lipids indicates that regulation of the fatty acid biosynthetic pathway, e.g. by use of desaturase and cyclooxygenase inhibitors, may be an approach to control pathogenesis. There are preliminary data to support this view (30,31). Interestingly, AA and EPA have been implicated in disease resistance as sesquiterpene phytoalexin elicitors (39). In conclusion, fatty acids and alcohols play multiple physiological and ecological roles, some of which are interchangeable, in *P. cactorum*. A determination of their relative importance with sterols as developmental triggers (cell-cycle mediated process [24]) vs fluidity modulators of mycelial membranes to initiate and then sustain growth and govern reproduction is under active investigation.

REFERENCES

1. Nes, W.R., and Nes, W.D. (1980) *Lipids in Evolution*, Plenum Press, New York, 224 pp.
2. Kerwin, J.L. (1987) in *Ecology and Metabolism of Plant Lipids* (Fuller, G., and Nes, W.D., eds.) pp. 329-342, ACS Symposium Series 325, Washington, D.C.
3. Wassef, M.K. (1977) *Adv. Lipid Res. 15*, 159-232.
4. Stafford, A.E., and Nes, W.D. (1983) *Exp. Mycol. 7*, 374-379.
5. Fuller, G., Nes, W.D., and Stafford, A.E. (1983) Composition and Biosynthesis of Fatty Acids of *Phytophthora cactorum*, paper presented at the 185th National ACS Meeting, Seattle, WA.
6. Nes, W.D., and Patterson, G.W. (1981) *J. Nat. Prod. 44*, 215-220.
7. Nes, W.D., and Stafford, A.E. (1983) *Proc. Natl. Acad. Sci. USA 80*, 3227-3231.
8. Nes, W.D., Le, P.H., Berg, L.R., Patterson, G.W., and Kerwin, J.L. (1986) *Experimentia 42*, 556-558.
9. Nes, W.D., Patterson, G.W., and Bean, G.A. (1979) *Lipids 14*, 458-462.
10. Nes, W.D., and Stafford, A.E. (1984) *Lipids 19*, 554-549.
11. Nes, W.D., and Bach, T.J. (1985) *Proc. Royal Soc. Series Lond. B 225*, 425-444.
12. Nes, W.D., and Heupel, R.C. (1986) *Arch. Biochem. Biophys. 224*, 211-217.
13. Nes, W.D., Dhanuka, I.C., and Pinto, W.J. (1986) *Lipids 21*, 102-106.
14. Salerno, L.F., and Parks, L.W. (1983) *Biochim. Biophys. Acta 752*, 240-243.
15. Nes, W.D., Campbell, B.C., Stafford, A.E., Haddon, W.F., and Benson, M. (1982) *Biochem. Biophys. Res. Commun. 108*, 1258-1263.
16. Lessire, R., Hartmann-Bouillon, M., and Cassagne, C. (1982) *Phytochemistry 21*, 55-59.
17. Lessire, R., Juguelin, H., Moreau, R., and Cassagne, C. (1985) *Phytochemistry 24*, 1187-1192.
18. Nes, W.D., Saunders, G.A., and Heftmann, E. (1981) *Lipids 16*, 744-748.
19. McMorris, T.C., and White, R.H. (1977) *Biochim. Biophys. Acta 486*, 308-312.
20. Elliott, C.G., and Knights, B.A. (1981) *Lipids 16*, 1-7.
21. Creamer, J.R., and Bostock, R.M. (1986) *Physiol. Mol. Plant Pathol. 28*, 215-225.
22. Kurantz, M.J., and Osman, S.F. (1983) *Physiol. Plant Pathol. 22*, 363-370.
23. Ko, W.H. (1985) *J. Gen. Microbiol. 131*, 2591-2594.
24. Nes, W.D. (1987) in *Ecology and Metabolism of Plant Lipids* (Fuller, G., and Nes, W.D., eds.) pp. 304-328, ACS Symposium Series 325, Washington, D.C.
25. Bahnweg, G. (1980) *Botan. Mar. 23*, 209-218.
26. Hohl, H.R. (1975) *Phytopathol. Z. 84*, 18-33.
27. Hendrix, J.W., Norman, C., and Apple, J.L. (1966) *Physiol. Plant Pathol. 19*, 159-166.
28. Hendrix, J.W., and Apple, J.L. (1964) *Phytopathology 54*, 987-994.
29. Ko, W.J., and Ho, W. (1983) *Ann. Phytopathol. Soc. Jpn. 49*, 316-321.
30. Kerwin, J.L., Simmons, C.A., and Washino, R.K. (1986) *Prostagland. Leuk. Med. 23*, 173-178.
31. Herman, R.P., and Herman, C.A. (1985) *Prostaglandins 29*, 819-828.
32. Hamberg, M., Herman, C.A., and Herman, R.P. (1986) *Biochim. Biophys. Acta 87*, 447-457.
33. Hamberg, M. (1986) *Biochim. Biophys. Acta 876*, 688-692.
34. Fox, N.C., Coniglio, J.G., and Wolf, F.T. (1983) *Exp. Mycol. 7*, 216-226.
35. Gellerman, J.L., and Schlenk, H. (1979) *Biochim. Biophys. Acta 573*, 23-30.
36. James, A.T. (1976) in *Function and Biosynthesis of Lipids* (Bazan, N.G., Brenner, R.R., and Giusto, N.M., eds.) pp. 51-74, Plenum Press, New York.
37. Heupel, R.C., Sauvaire, Y., Le, P.H., Parish, E.J., and Nes, W.D. (1986) *Lipids 21*, 69-75.
39. Kuc, J., Tjamos, E., and Bostock, R. (1984) in *Isopentenoids in Plants: Biochemistry and Function* (Nes, W.D., Fuller, G., and Tsai, L., eds.) pp. 103-126, Dekker, New York.

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Neutral Lipid Transfer Protein Does Not Regulate α-Tocopherol Transfer Between Human Plasma Lipoproteins

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Vitamin E has no known plasma carrier protein and is transported by plasma lipoproteins. The site of association of vitamin E in the lipoprotein particle and the mode of transfer of vitamin E between plasma lipoproteins have not been ascertained. Since neutral lipids (triglycerides and cholesterol esters) exchange between plasma lipoproteins by processes mediated by neutral lipid transfer protein, we questioned that if vitamin E, a hydrophobic molecule, is carried in the core of the lipoprotein particle then its transfer between plasma lipoproteins may be mediated by neutral lipid transfer protein. Transfer of D-α(5-methyl-^{3}H)tocopherol from in vitro-labeled human plasma lipoprotein fractions to other plasma lipoproteins was measured under incubation conditions that were designed to yield markedly differing degrees of neutral lipid exchange. Despite the presence of the d > 1.21 g/ml lipoprotein-poor plasma fraction or purified lipid transfer protein that resulted in up to a 10-fold increase in neutral lipid transfer, vitamin E transfer between very low density lipoproteins, low density and high density lipoproteins remained constant. Even excess amounts of lipid transfer protein, which caused triglyceride transfer between very low density and high density lipoproteins to reach saturation, failed to affect significantly vitamin E transfer. Vitamin E distribution between lipoprotein fractions did correlate with lipoprotein mass ratios. Vitamin E transfer was higher as the protein ratio of acceptor lipoproteins to donor lipoproteins increased. We conclude that vitamin E transfer between lipoproteins is not dependent primarily on neutral lipid transfer protein and is not mediated via neutral lipid transfer.
Lipids 23, 17-21 (1988).

α-Tocopherol is a lipid soluble vitamin that has no known plasma carrier protein and is transported by plasma lipoproteins (1,2). There is disagreement concerning the relative amounts of α-tocopherol in the different lipoprotein fractions. Various studies (3,4) have concluded that the low density lipoproteins (LDL) are the major carriers of tocopherol, and only when high levels of plasma tocopherol are present do the other lipoproteins carry proportionally increased amounts (5). Bjornson et al. (4) have observed that vitamin E is associated primarily with the lipoprotein class that contains the largest amount of total lipid. Yet, others have reported that vitamin E is transported mainly in high density lipoproteins (HDL) (6,7), and tocopherol levels correlate with HDL protein levels, suggesting that the apoproteins in HDL also may bind the vitamin (2).

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Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoproteins; HDL, high density lipoproteins; TG, triglyceride; CE, cholesterol ester; LTP, neutral lipid transfer protein.

Vitamin E is exchanged readily among lipoproteins (8) and between lipoproteins, red blood cells (9) and tissue membranes (10). Transfer of lipoprotein components among lipoproteins and cellular compartments is determined, at least in part, by their relative hydrophobicity. Cholesterol spontaneously transfers by a aqueous diffusion mechanism (11). α-Tocopherol, another amphiphilic compound, has been shown to transfer both spontaneously between liposomes (12) and by a factor present in rat liver cytosol that enhances its transfer between membranes (10,13). Cholesterol ester and triglyceride, which are more hydrophobic, require specific plasma proteins to mediate transfer.

The exchange protein that transfers cholesterol esters (CE) and triglyceride (TG) according to the relative mass of these lipids in the different lipoproteins particles recently has been isolated and characterized (14-16). This neutral lipid transfer protein is present in the d > 1.21 g/ml lipoprotein-poor plasma fraction. A reversible mass transfer of TG and CE, mediated by neutral lipid transfer protein, has been observed to occur between very low density lipoproteins (VLDL) and either LDL or HDL, both in vivo and in vitro (17-20), resulting in transfer of TG from the triglyceride-rich lipoproteins (VLDL) to LDL or HDL with a concomitant loss of CE from the cholesterol ester-rich lipoproteins and enrichment of VLDL with cholesterol ester. No such specific transfer protein has been observed yet for the transfer of α-tocopherol between plasma lipoproteins.

Tocopherol equilibrates between lipoproteins at a rate that is 2-3 times slower than that of cholesterol and its distribution correlates best with the total lipid mass of the lipoproteins (8), suggesting an association of vitamin E with the non-polar triglyceride/cholesterol ester particle core. We hypothesized that if vitamin E is carried mainly in the core of the lipoprotein particle, then the transfer of tocopherol between plasma lipoproteins may be mediated by neutral lipid transfer protein. Therefore, to better understand the association of vitamin E with lipoprotein particles and further characterize the partitioning of tocopherol between plasma lipoproteins we have studied the role of neutral lipid transfer protein in the transfer of tocopherol among human plasma lipoproteins.

MATERIALS AND METHODS

Vitamin E. D-α(5-methyl-^{3}H)tocopherol at a specific radioactivity of 20.2 mCi/mg and radiochemical concentration of 1.0 mCi/ml, was obtained from Amersham International (Bucks., England) and purified by thin layer chromatography on silica gel plates using benzene-absolute ethanol 99:1. The vitamin was stored at −20 C and rechecked periodically by thin layer chromatography to maintain purity >95%.

Preparation of plasma lipoproteins labeled with vitamin E. Plasma lipoproteins were labeled as described by

Bjornson et al. (9): 25 ml flasks were silanized using silicone coating Sigmacote from Sigma Chemical Co. (St. Louis, MO). The flasks were dried under nitrogen. Radioactive tocopherol (2 μl; 1.6×10^6 dpm ^{3}H/1 μl) was added to the silanized flask in 0.5 ml of benzene and evaporated to a thin film by rotating the flask under nitrogen at room temperature. Five ml of fresh human plasma containing 1 mg/ml ethylenediaminetetraacetic acid (obtained from healthy, fasting donors) was added and incubated at 37 C for 12 hr in a shaking water bath. Vitamin E-labeled plasma lipoproteins then were separated from this plasma by sequential salt density ultracentrifugation (21). Individual lipoprotein fractions (separated from human plasma by sequential salt density ultracentrifugation VLDL at d < 1.019, LDL at d < 1.063, HDL at d < 1.21 g/ml) similarly were labeled with radioactive vitamin E. Incorporation of the isotope into the lipoprotein fractions using either one of the above methods of labeling ranged from 30–45% of the initial amount of radioactivity. The method of labeling did not affect the results obtained.

Lipid transfer protein (LTP) was prepared as described by Albers et al. (15), using a modification of the method of Pattnaik et al. (14). In brief, the d = 1.21–1.25 g/ml plasma fraction was placed on a phenyl-Sepharose column. The fraction containing transfer activity was further purified by diethylaminoethyl anion exchange chromatography. Lipid transfer activity was assayed by measuring the transfer of [^{3}H]cholesterol ester from [^{3}H]cholesterol ester-labeled HDL to the d < 1.019 g/ml plasma fraction.

The following incubation systems were studied (the fraction marked with an asterisk is labeled with vitamin E): VLDL* + LDL; VLDL + LDL*; VLDL* + HDL; VLDL + HDL*; LDL* + HDL; LDL + HDL*. Incubations were performed under the following conditions in the presence of either human d > 1.21 g/ml lipoprotein-poor plasma, purified neutral lipid transfer protein, rat d > 1.21 g/ml lipoprotein poor plasma (rat plasma is devoid of neutral lipid transfer activity), albumin (fatty acid-free, Sigma bovine albumin), or 50 mM Tris, 0.15 M NaCl, 0.05% EDTA, pH 7.4. The concentration of albumin or of the protein in the lipoprotein-poor plasma fraction was adjusted to a concentration of 4–5 g% in each system. Most incubations were performed at 37 C for 18 hr. Individual experiments were performed at 4 C. LTP-mediated CE-TG exchange previously has been shown by us to be minimal at 4 C (19).

Incubations of VLDL + HDL* also were performed using VLDL labeled with ^{14}C-triglyceride. (^{14}C-triglyceride)VLDL was prepared by incubation of ^{14}C-triolein Intralipid 4 Ci/ml with human VLDL at 37 C for 18 hr in the presence of d > 1.21 g/ml lipoprotein-poor plasma. Prior to the incubation, Intralipid was washed in order to rid Intralipid of excess phospholipid. Washed Intralipid was prepared by repeated centrifugation of Intralipid suspended in 0.15 M NaCl, 0.05% EDTA as described (22). This procedure resulted in a decrease from 12.5% to 3.4% in the relative weight composition of phospholipid in Intralipid. ^{14}C-triglyceride labeled VLDL was separated by ultracentrifugation at d < 1.019 g/ml after flotation of the Intralipid at d = 1.006 g/ml by swinging bucket ultracentrifugation at 25,000 rpm for 15 min. The lipoprotein fraction ratios in the incubations usually were adjusted so that the triglyceride in the triglyceride-rich lipoprotein was five-fold greater than the cholesterol ester in the cholesterol ester-rich lipoprotein (a ratio of 5:1 previously has been shown to be practical for monitoring neutral lipid mass transfer processes [19]). In addition, some incubations were performed at different protein ratios. Following each incubation, sequential salt density ultracentrifugation was used to separate the fractions corresponding to d < 1.019 g/ml-modified, "M"-VLDL; d = 1.019–1.063 g/ml-modified, "M"-LDL; d = 1.063–1.21 g/ml-modified, "M"-HDL. "M" denotes the modified lipoprotein fraction in terms of TG/CE composition following mass transfer.

In some incubations of LDL + HDL, separation of lipoprotein fractions was performed by precipitation with $MnCl_2$ and heparin, as described by Burstein et al. (23).

The total radioactivity in each fraction was calculated and, accordingly, the net transfer of radiolabeled vitamin E between the lipoprotein fractions determined.

Chemical analysis. Total and free cholesterol were assayed using the Test Combination Cholesterol Kit of Boehringer Mannheim (Mannheim, Germany). Triglycerides were assayed by the RapidChem enzymatic triglycerides reagent kit of Macomb Biotechnology Inc. (Mt. Clemens, MI). Protein was measured by the method of Lowry et al. (24) with bovine serum albumin as a standard.

RESULTS

In all incubation systems studied, vitamin E transfer from one lipoprotein fraction to another did not correlate with transfer of neutral lipids. Although in each system incubation conditions varied so that neutral lipid transfer increased up to 10-fold, vitamin E transfer showed no real changes. Figure 1 depicts experiments designed to obtain widely differing neutral lipid exchange. Although neutral lipid transfer varied considerably, the transfer of radiolabeled vitamin E from LDL to VLDL remained relatively constant in incubations of LDL with VLDL (VLDL-TG:LDL-CE 5:1) under five different conditions: in the presence of saline, d > 1.21 g/ml lipoprotein-poor plasma, purified lipid transfer protein and either albumin or rat

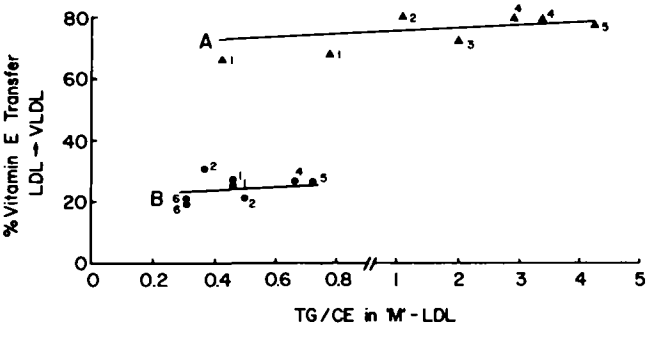

FIG. 1. Vitamin E transfer from LDL to VLDL at different ratios of neutral lipid exchange. VLDL and LDL (^{3}H-tocopherol) were incubated at VLDL:LDL protein ratios of A, 1.5:1 (▲) and B, 0.3:1 (●). Each set of incubations was performed in the presence of albumin (1), saline (2), d > 1.21 g/ml lipoprotein-poor plasma at 4 C (3) and at 37 C (5), neutral lipid transfer protein at 4 C (6) and 37 C (4). Neutral lipid exchange is measured by TG/CE ratios in "M"-LDL. Each point represents the mean of two separate sets of experiments.

lipoprotein-poor plasma. Specifically in the presence of either d > 1.21 g/ml lipoprotein-poor plasma or purified LTP, neutral lipid transfer resulted in an 8-11-fold increase in the TG/CE ratios in LDL after incubations. In contrast, in incubations in the presence of saline, albumin or rat lipoprotein-poor plasma little or no neutral lipid transfer occurred; the TG/CE ratio in post-incubation LDL remained relatively low, close to native LDL. Under all the above conditions, the transfer of vitamin E from LDL to VLDL was 60-80% of the initial amount of vitamin E in VLDL. In a similar set of incubations in which the ratio of VLDL-TG to LDL-CE was 1:1 (protein ratio 0.3:1), vitamin E transfer from LDL to VLDL was 20-30% (Fig. 1) and similarly was independent of the degree of triglyceride and cholesterol ester transfer in the system.

In incubation systems in which VLDL was the lipoprotein fraction labeled with vitamin E, transfer of vitamin E from VLDL to LDL was approximately 10% in the presence of either saline or d > 1.21 g/ml lipoprotein poor plasma although neutral lipid transfer was almost threefold higher in the presence of the lipoprotein-poor plasma fraction (Fig. 2).

To determine if excess amounts of transfer protein might affect vitamin E transfer, incubations were performed with amounts of transfer protein that produced no further increase in TG/CE exchange. The transfer of [^{3}H]α-tocopherol and [^{14}C]triglyceride between vitamin E-labeled HDL and triglyceride-labeled VLDL were measured. Vitamin E transfer did not correlate significantly with neutral lipid transfer. With increasing concentrations of purified lipid transfer protein, transfer of vitamin E remained relatively constant (40-55%) even when the increase in triglyceride transfer (from 20% to 80%) reached saturation at the higher lipid transfer protein concentrations (Fig. 3).

Vitamin E transfer between lipoprotein fractions was found to correlate with mass ratios of the donor lipoprotein to acceptor lipoprotein. This was observed in all the incubation systems studied. Vitamin E transfer from VLDL to HDL increased with rising HDL protein to VLDL protein ratios with a correlation coefficient of r = 0.87 (Fig. 4). A similar effect was demonstrated with VLDL and LDL (data not shown).

An especially high affinity of vitamin E to HDL was shown in incubations of HDL and LDL (Fig. 5). Despite conditions in which LDL lipid mass was up to 10-fold that of HDL, most of the vitamin E remained associated with HDL. Only at LDL/HDL lipid ratios 15-16:1 did most of the vitamin E associate with LDL. This implies that factors other than simple lipid mass ratios influence partitioning of vitamin E between plasma lipoproteins.

The effect of LTP on transfer of vitamin E between lipoproteins was studied following 18-hr incubations, a time period previously shown (19) to be necessary for achieving substantial changes in lipid mass and for reaching an equilibrium of mass transfer. To ascertain that an earlier effect of LTP was not being masked by measurements at 18 hr, we performed incubations at increasing time intervals (Fig. 6). There was no consistent difference in vitamin E transfer between lipoproteins with or without LTP, even at very short incubation periods.

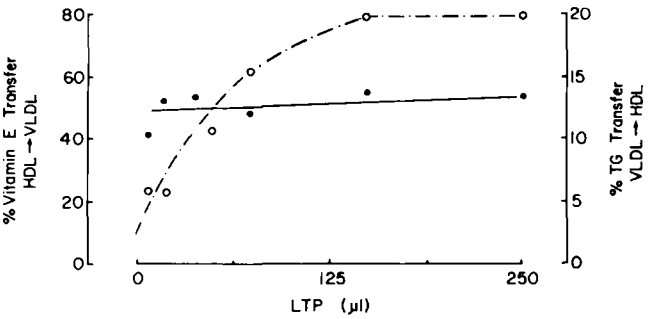

FIG. 3. The effect of neutral lipid transfer protein on vitamin E and triglyceride transfer between VLDL and HDL. VLDL (^{14}C-triglyceride) and HDL (^{3}H-tocopherol) were incubated in the presence of increased concentrations of neutral lipid transfer protein. Each point represents the mean of two separate sets of experiments. ●—●, ^{3}H-tocopherol transfer. ○—○, ^{14}C-triglyceride transfer.

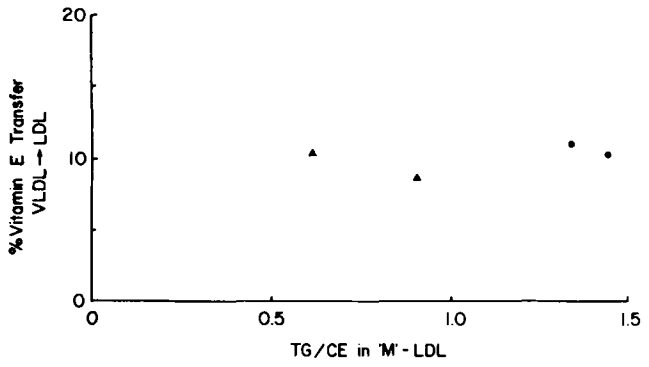

FIG. 2. Vitamin E transfer from VLDL to LDL at different ratios of neutral lipid exchange. VLDL (^{3}H-tocopherol) and LDL were incubated at VLDL:LDL protein ratio of 1.5:1. Incubations were performed in the presence of d > 1.21 g/ml lipoprotein-poor plasma (●) and saline (▲). Neutral lipid exchange is measured by TG/CE ratios in "M"-LDL. Each point represents the mean of two separate sets of experiments.

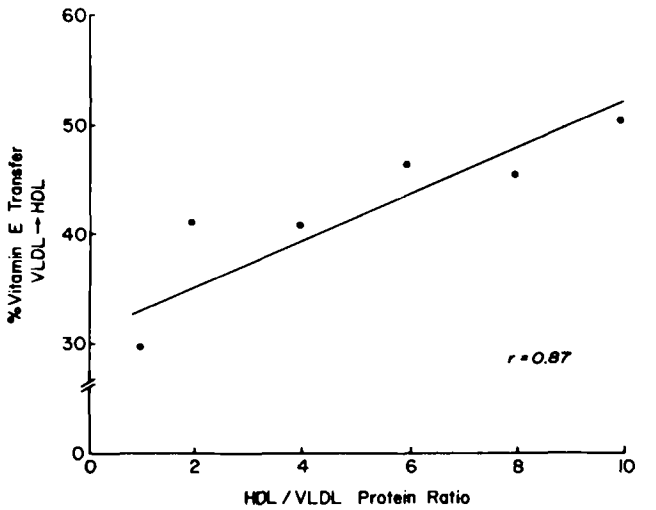

FIG. 4. The effect of lipoprotein mass ratios on vitamin E transfer between VLDL and HDL. VLDL (^{3}H-tocopherol) and HDL were incubated at increasing HDL:VLDL protein ratios. Incubations were performed in the presence of d > 1.21 g/ml lipoprotein-poor plasma. Each point represents the mean of two separate sets of experiments.

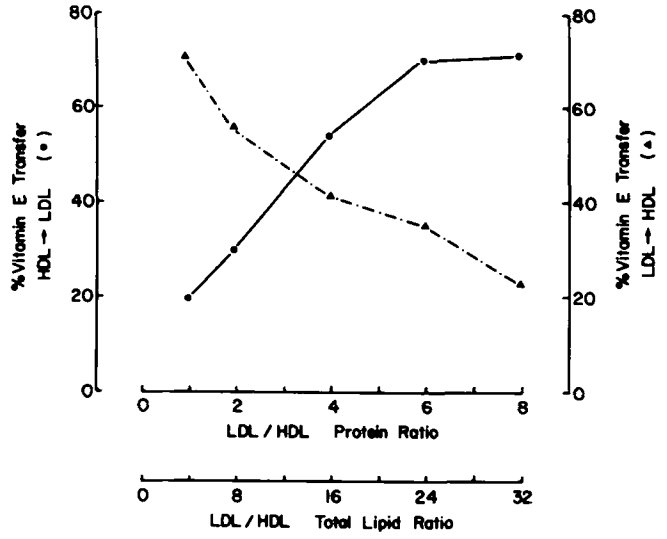

FIG. 5. The effect of lipoprotein mass ratios on vitamin E transfer between HDL and LDL. Incubations of HDL (^{3}H-tocopherol) and LDL; (●) or LDL (^{3}H-tocopherol) and HDL; (▲) were performed at varying LDL:HDL mass ratios. Incubations were performed in the presence of d > 1.21 g/ml lipoprotein-poor plasma. Each point represents the mean of two separate sets of experiments.

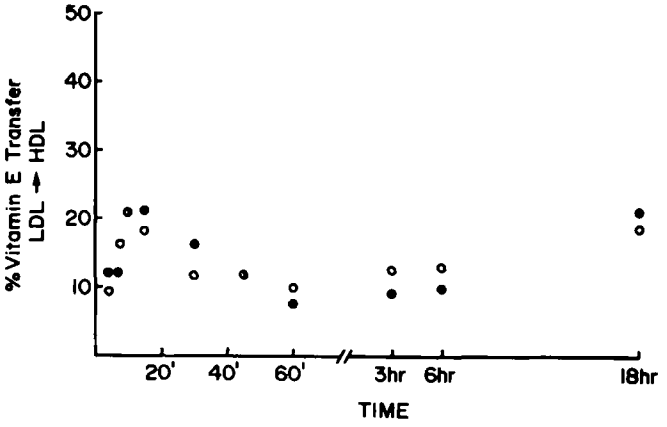

FIG. 6. Time course of vitamin E transfer between LDL and HDL with and without LTP. Incubations of LDL (^{3}H-tocopherol) and HDL (LDL:HDL protein ration 1:1) were performed at increasing time intervals in the absence of and presence of d > 1.21 g/ml lipoprotein-poor plasma. Each point represents the mean of two separate sets of experiments.

DISCUSSION

α-Tocopherol, a lipid-soluble vitamin, is transported in plasma primarily by the lipoprotein class that contains the largest amount of total lipid. α-Tocopherol levels in each lipoprotein fraction have been shown to correlate better with the amount of free cholesterol in the lipoprotein particle than with any other individual lipid (4). Earlier work on the partitioning of free cholesterol in plasma lipoproteins has suggested that most free cholesterol is associated with surface components rather than core lipids (25). But tocopherol equilibrates between lipoproteins at a rate that is slower than that of cholesterol; on isolated surface components of VLDL, two-thirds of the associated free cholesterol but only one-third of the tocopherol are separated with the surface phases (9). This suggests that up to 2/3 of lipoprotein vitamin E is associated with core lipids and, if so, may be expected to participate in exchange processes similar to those of cholesterol ester and triglyceride. Lipoprotein lipase has been shown by Traber et al. (26) to mediate the binding of vitamin E from chylomicrons to cell membranes. Lipoprotein lipase similarly enhances binding and possibly uptake of CE from lipoproteins to cells (27). The fact that CE and vitamin E may share a common mechanism of cellular uptake further suggested a close association of vitamin E with CE in the lipoprotein particle. CE transfer between lipoproteins is mediated by LTP and additionally, LTP recently has been shown to enhance the uptake of HDL-CE into liver and smooth muscle cells (28). Therefore, ascertaining a role for LTP in the transfer of vitamin E between lipoproteins also might have contributed to our understanding of the mechanism of uptake of plasma vitamin E by tissues.

Despite the evidence that substantial amounts of tocopherol are associated with the core of the lipoprotein particle, we have failed to demonstrate that tocopherol transfers between lipoproteins in conjunction with the transfer of the major core lipids, triglycerides and cholesterol esters. Under conditions leading to a wide range of cholesterol ester-triglyceride transfers, vitamin E transfer remained relatively constant. In the presence of lipoprotein-poor plasma or purified lipid transfer protein, up to a 10-fold change in the ratio of TG/CE in modified lipoproteins was achieved as compared to incubations in the presence of saline, albumin or rat plasma, which has no neutral lipid transfer activity. Yet vitamin E transfer was similar under all these conditions (Figs. 1-3). A possible cause for the lack of effect of LTP on vitamin E transfer may be a spontaneous rapid vitamin E exchange between lipoproteins that overshadows the slower transfer due to LTP. This could occur by rapid core-surface partitioning of the vitamin followed by equilibration between lipoproteins of the vitamin E partitioned in the particle surface.

Vitamin E distribution between lipoprotein fractions did correlate with lipoprotein:lipid and/or protein ratios. This was shown in incubation systems of VLDL-LDL, VLDL-HDL and LDL-HDL. Nevertheless, we noted that vitamin E transfer does not behave solely or primarily in accordance with lipoprotein total lipid mass in view of results observed in incubations of LDL with HDL. At LDL:HDL protein ratios of 2:1, the lipid ratio is approximately 8:1 but despite this high LDL total lipid mass, vitamin E was distributed so that it was predominantly associated with HDL (Fig. 5). This partitioning was observed irrespective of which lipoprotein fraction was the one initially labeled with vitamin E. These results are of interest in light of Behrens' (29) recent observations on the distribution of tocopherol in plasma lipoproteins, noting a high correlation between α-tocopherol and HDL-protein but such correlations were not observed for LDL. Thus, the factors determining partitioning of vitamin E between lipoproteins and in the lipoprotein particle itself are complex and do not depend only on total lipid mass ratios. Structural properties and specific lipid-protein interactions may prove important. Further studies will

clarify the precise partitioning of vitamin E in the lipoprotein particle and its mechanisms of transfer between human plasma lipoproteins.

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REFERENCES

1. McCormick, E.C., Cornwell, D.G., and Brown, J.B. (1960) *J. Lipid Res. 1,* 221-228.
2. Behrens, W.A., Thompson, J.N., and Madere, R. (1982) *Am. J. Clin. Nutr. 35,* 691-696.
3. Rubinstein, H.M., Dietz, A.A., and Srinavasan. R. (1969) *Clin. Chim. Acta 23,* 1-6.
4. Bjornson, L.K., Kayden, H.J., Miller, E., and Moshell, A.N. (1976) *J. Lipid Res. 17,* 343-352.
5. Kayden, H.J., and Bjornson, L. (1972) *Ann. N.Y. Acad. Sci. 203,* 127-140.
6. Lewis, L.A., Quaife, M.L., and Page, I.H. (1954) *Am. J. Physiol. 178,* 221-222.
7. Takahashi, Y., Urono, K., and Kimura, S. (1977) *J. Nutr. Sci. Vitaminol. 23,* 201-209.
8. Massey, J.B. (1984) *Biochim. Biophys. Acta 793,* 387-392.
9. Bjornson, L.K., Gniewkowski, C., and Kayden, H.J. (1975) *J. Lipid Res. 16,* 39-53.
10. Murphy, D.J., and Mavis, R.D. (1981) *J. Biol. Chem. 256,* 10464-10468.
11. McLean, L.R., and Phillips, M.C. (1981) *Biochemistry 20,* 2893-2900.
12. Nakagawa, Y., Nojima, S., and Inoue, K. (1980) *J. Biochem. 87,* 497-502.
13. Mowri, H., Nakagawa, Y., Inoue, K., and Nojima, S. (1981) *Eur. J. Biochem. 117,* 537-542.
14. Pattnaik, N.M., Montes, A., Hughes, L.B., and Zilversmit, D.B. (1978) *Biochim. Biophys. Acta 530,* 428-438.
15. Albers, J.J., Tollefson, J.H., Chen, C., and Steinmetz, A. (1984) *Arteriosclerosis 4,* 49-58.
16. Tall, A.R., Abreu, E., and Shuman, J. (1983) *J. Biol. Chem. 258,* 2174-2180.
17. Nichols, A.V., and Smith, L. (1965) *J. Lipid Res. 6,* 206-210.
18. Quarfordt, S.H., Boston, F., and Hilderman, H. (1971) *Biochim. Biophys. Acta 231,* 290-294.
19. Deckelbaum, R.J., Eisenberg, S., Oschry, Y., Butbul, E., Sharon, I., and Olivecrona, T. (1982) *J. Biol. Chem. 257,* 6509-6517.
20. Deckelbaum, R.J., Eisenberg, S., Oschry, Y., Granot, E., Sharon, I., and Bengtsson-Olivecrona, G. (1986) *J. Biol. Chem. 261,* 5201-5208.
21. Havel, R.J., Eder, A.H., and Bragdon, J.H. (1955) *J. Clin. Invest. 34,* 1345-1353.
22. Granot, E., Deckelbaum, R.J., Eisenberg, S., Oschry, Y., and Bengtsson-Olivecrona, G. (1985) *Biochim. Biophys. Acta 833,* 308-315.
23. Burstein, M., Scholnick, H.R., and Morfin, R. (1970) *J. Lipid Res. II,* 583-595.
24. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
25. Deckelbaum, R.J., Shipley, G.G., and Small, D.M. (1977) *J. Biol. Chem. 252,* 744-754.
26. Traber, M.G., Olivecrona, T., and Kayden, H.J. (1985) *J. Clin. Invest. 75,* 1729-1734.
27. Stein, O., Halperin, G., Leitersdorf, E., Olivecrona, T., and STein, Y. (1984) *Biochim. Biophys. Acta 795,* 47-59.
28. Granot, E., Tall, A., and Tabas, I. (1987) *J. Biol. Chem. 262,* 3482-3487.
29. Behrens, W.A., and Madere, R. (1985) *Nutr. Res. 5,* 167-174.

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A Novel Method to Administer Radiolabeled Lipid to Juvenile Oysters

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Particles prepared from egg yolk were shown to encapsulate protein and to be in a size range that would be filtered by the oyster. A radiotracer study involving the addition of radiolabeled phosphatidylcholine to egg yolk demonstrated that the egg yolk particles were taken up and metabolized by juvenile oysters *(Crassostrea gigas).* Catabolism of the radiolabeled lipid and subsequent resynthesis into non-lipid components occurred to a slight extent. The main factor responsible for the distribution of radioactivity amongst the lipids in the stomach tissue was believed to be transacylation.
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Lipids play an important role in the diet and physiology of marine bivalves (1–4). While the lipid composition of bivalves has been detailed extensively (5), few experiments have been made to determine their metabolic pathways.

Several methods have been used to deliver radiolabeled lipids or lipid precursors to bivalves. Trider and Castell (3) incubated oyster gill and mantle tissue in a solution containing ^{14}C-acetate. Allen and Conley (6) administered 1-^{14}C-palmitate to oysters by means of a blunted hypodermic needle placed in the stomach. Lubet et al. (7) injected the mussle foot with 1-^{3}H-glycerol. Lipid metabolism measured using these means of incorporation may differ from the metabolism that occurs in bivalves in their natural habitat.

In the natural habitat, the majority of lipid and lipid precursors for bivalves are obtained by filtering and ingesting algae. Taking this into account, to monitor the fate of labeled linolenic acid and higher homologues clams have been fed a culture of *Phaeodactylum tricornutum* previously incubated with 1-^{14}C linolenic acid (8). In addition, juvenile oysters were fed either *Tetraselmis suecia* or *Dunaliella tertiolecta* that were grown in the presence of ^{14}C-sodium bicarbonate for 24 hr (4). However, the main limitation of using radiolabeled algae is the difficulty of following the metabolism of one specific lipid component.

Like algae, liposomes prepared from purified lipid are particulate and are filtered and ingested by the oysters. The metabolism of ^{14}C-dipalmityl phosphatidylcholine was followed in oysters fed liposomes that contained the radiolabeled lipid (9). Other nutritional components such as protein or carbohydrate, which are lacking in liposomes, may modify the metabolism of ingested lipid. A suitable alternative for the source material of liposomes is to use egg yolk. With one of the key components in egg yolk being lipid, the idea was generated that vesicles that would be similar to liposomes could be prepared. This paper describes the metabolic fate of phosphatidylcholine, L-α-1-palmitoyl-2-arachidonyl [arachidonyl-1-^{14}C] (^{14}C-PAPC) when the radiolabeled lipid is incorporated into egg yolk particles and fed to oysters.

MATERIALS AND METHODS

Preparation of fluorescent liposomes. Fluorescent liposomes were prepared with 10.5 mg of egg phosphatidylcholine (PC)/cholesterol/stearylamine, 87:5:8 (P-L Biochemicals, Inc., Milwaukee, WI). The material was evaporated from chloroform to a thin film on the wall of a round-bottom flask. Five ml of a solution of 30 ppt synthetic seawater, Instant Ocean from Aquarium Systems (Mentor, OH), containing 1 mg fluorescein isothiocyanate bovine serum albumin (FITC-BSA) and several glass beads were added, and the flask swirled for one hour. Unentrapped FITC-BSA was removed by centrifuging the liposomes in an IEC International clinical centrifuge (315 × g) and replacing the supernatant with Instant Ocean from Aquarium Systems Inc. (Eastlake, OH). All solutions of Instant Ocean used in this study were at 30 g Instant Ocean/l of deionized water. After several repetitions, no fluorescence was observed in the filtrate. The vesicles were diluted to 200 ml with Instant Ocean.

Preparation of fluorescent egg yolk particles. Fluorescent egg yolk particles were prepared by placing 0.20 gm of fresh egg yolk (containing 9.7% phospholipids) in a 30 ml homogenizing tube and adding 20 ml of a solution of 30 ppt Instant Ocean containing FITC-BSA (10 mg), corn starch (96 mg), thiamin-HCl (3.9 μg), riboflavin (5.8 μg), niacin (39 μg), D-biotin (0.4 μg), Na-pantothenate (9.7 μg), pyridoxine (3.9 μg), folic acid (1.9 μg), vitamin B_{12} (0.04 μg), myo-inositol (80 μg) and ascorbic acid (29 μg). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The materials were mixed with a high speed tissue homogenizer for 10 to 20 seconds. Unentrapped FITC-BSA was removed by centrifuging (315 × g) the egg yolk particles in an IEC International clinical centrifuge and replacing the supernatant with Instant Ocean. After several repetitions, no fluorescence was observed in the filtrate, and the final volume was brought to 200 ml with Instant Ocean. Since it was beyond the scope of this study to determine the physical nature of the lipid material formed, we will refer to the material as particles. However, it is probable that due to the high triacylglycerol content of egg yolk, the material is a mixture of vesicles and micelles containing triacylglycerol.

Preparation of radiolabeled egg yolk particles. Radiolabeled egg yolk particles were prepared by placing 0.21 g of fresh egg yolk and 10 μC of ^{14}C-PAPC (New England Nuclear, Boston, MA) in a 30 ml homogenizing tube. After adding 21.0 ml of Instant Ocean containing 0.105 g starch, the solution was mixed for one min with a high speed tissue homogenizer. The solution then was diluted with Instant Ocean to a final volume of 210 ml.

Feeding and rearing of oysters. Juvenile Pacific oysters, *C. gigas*, weighing 1.5 to 2.0 g, were obtained from Ted

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Abbreviations: AA, arachidonic acid; CL, cardiolipin; CP, choline phospholipids; ^{14}C-DPPC, di[1-^{14}C] palmitoyl phosphatidycholine; EP, ethanolamine phospholipids; FITC-BSA, fluorescein isothiocyanate bovine serum albumin; NL, neutral lipids; PC, phosphatidylcholine; ^{14}C-PAPC, phosphatidylcholine, L-α-1-palmitoyl-2-arachididonyl [arachidonyl-1-^{14}C]; PPL, phosphonolipid; SP, serine phospholipids; TLC, thin layer chromatography.

Kuiper in Eureka, CA. The oysters were placed on a screen mesh two inches above the bottom of a large bucket containing 20 l of Instant Ocean solution at 19 C. Once a day for a month the oysters were fed unlabeled egg yolk particles.

For the radiotracer study, 80 oysters were starved for 24 hr and were placed on a plastic tray (1.0 mm mesh) suspended one inch above the bottom of a dish pan (26 cm × 36 cm) that contained seven liters of Instant Ocean. The water was maintained at 19 C in a temperature-controlled room. An airlift pump and three airstones attached to a Whisper 800 air pump were used to aerate and circulate the water. Oysters were fed the radiolabeled egg yolk particles twice a day for three days. After transfer to fresh Instant Ocean, the oysters were fed unlabeled egg yolk particles twice a day for five days.

Analysis of radiolabeled tissue and egg yolk. Analysis of oysters consisted of removing 24 oysters from the container after zero, one, three and eight days. Assuming equal access of oysters to the radiolabeled food, each oyster from day 1 had been exposed to 1.53×10^5 dpm, and each oyster from days 3 and 8 had been exposed to 3.71×10^5 dpm. Each set of 24 oysters was divided into two groups. After each oyster was weighed, it was dissected. The mantle, muscle and gills of each group were pooled and weighed. In addition, the digestive tissues of each group were pooled and weighed. Moisture content was determined by lyophilization of the pooled tissues.

Lipid extraction of the lyophilized tissue and radiolabeled egg yolk particles was performed according to the method of Folch et al. (10). Material insoluble in the chloroform-methanol phase was collected on a column packed with glass wool, digested in NCS solubilizer (Amersham, Arlington Heights, IL) and counted for radioactivity using Bray's scintillation solvent system (11). Samples of both aqueous phase and lipid extract also were measured for radioactivity using the liquid scintillation medium 3a70B (Research Products International Corp., Mount Prospect, IL).

Two-dimensional thin layer chromatograph (TLC) was used to separate individual phospholipids on Silica Gel H plates (0.25 mm thickness). The first dimension solvent system consisted of chloroform/methanol/ammonia (65:25:4, v/v/v). The second dimension solvent system consisted of chloroform/methanol/ammonia (100:50:12, v/v/v). Exposure to iodine revealed the location of neutral lipids (NL) and each phospholipid. All phospholipids with the exception of phosphonolipid (PPL) were identified by comparing to purified phospholipid standards subjected to the same solvent systems. The PPL spot tentatively was identified by the procedure of Stillway and Harmon (12).

Quantitation of individual phospholipids from the day 0 samples was accomplished by the phosphorus assay of Bartlett (13). Individual phospholipids from radiolabeled lipid samples were scraped into vials, hydrated with water and counted for radioactivity in 3a70B.

Neutral lipids from radiolabeled samples were separated on Silica Gel H plates (0.25 mm thickness) by one-dimensional TLC. The solvent system employed was hexane/ethyl ether/glacial acetic acid (80:20:1, v/v/v). Bands 0.5 cm in width were scraped into vials, hydrated with water and counted for radioactivity in 3a70B.

Quantitation of triacylglycerols from the 0 day samples was accomplished by applying each lipid sample to Silica Gel H plates prepared with 0.05% sodium fluorescein. After running the plate in the neutral solvent system described above, the triacylglycerol spot was detected under fluorescent light and scraped into a test tube. After eluting the triacylglycerols from the silica gel with chloroform, the samples were evaporated to dryness and then quantified according to the procedure of Van Handel and Zilversmit (14).

Total lipid from the mantle, gill and muscle tissue and total lipid from the digestive tissues were analyzed for distribution of radioactivity between the acyl portion and the glycerol-base backbone. A portion of each lipid sample was hydrolyzed with 4% H_2SO_4, and the solution was extracted with hexane. Samples of both the aqueous and organic phases were taken for liquid scintillation counting.

The fatty acid compositions of NL and of each phospholipid from the digestive tissues and from the gill, mantle and muscle tissues were determined for day 0 oysters. After separation in two dimensions on fluorescent silica gel plates, the lipids were subjected to methanolysis for 90 min in 4% H_2SO_4 in methanol (90 C). The methyl esters formed were extracted with hexane and dried over anhydrous sodium sulfate. The fractions were purified and the dimethyl acetals separated from the methyl esters on thin-layer plates (Silica Gel H) developed in benzene (15).

Gas liquid chromatography of the esterified fatty acids was done with a Varian Aerograph Model 1200 gas chromatograph equipped with a Hewlett-Packard 3380A computing integrator. The column was packed with 10% SP-2330 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, PA). Chromatographic peaks were identified by comparison of their relative retention times with those of standards subjected to the same conditions.

RESULTS AND DISCUSSION

Ingestion of fluorescent egg yolk particles. FITC-BSA-containing liposomes shown in Figure 1A resembled those of liposomes prepared by Parker and Selivonchick (9). Egg yolk particles (Fig. 1B) prepared with FITC-BSA were similar in shape and size to these liposomes. Most of the fluorescence appeared to be intravesicular, indicating that the protein was encapsulated within the particle. Absorption of protein to the outside of the vesicle may have occurred slightly. However, the high ionic strength of the synthetic seawater should have reduced the absorption of protein (16).

Analysis of lipids from oyster tissue. Initial analysis of oyster tissues revealed that approximately 81% of the weight was water (Table 1). These values corresponded to those obtained with pre-starved *C. gigas* (17). The recovery of lipid from the total oyster agreed closely to recoveries obtained by Watanabe and Ackman (18) for *Crassostrea virginica* and *Ostrea edulis.*

Less than 10% of the total lipid from the oysters was found in the triacylglycerol fraction. In contrast, *C. virginica* and *O. edulis* have been reported to contain at least 20% of their lipid as triacylglycerol (18). For *C. gigas,* Allen and Conley (6) reported almost 27% of the total lipid as triacylglycerols. As oysters showed decreased triacylglycerol levels when under physiological stress (1), the low content of triacylglycerol found in this study would suggest that the nutritional state of the

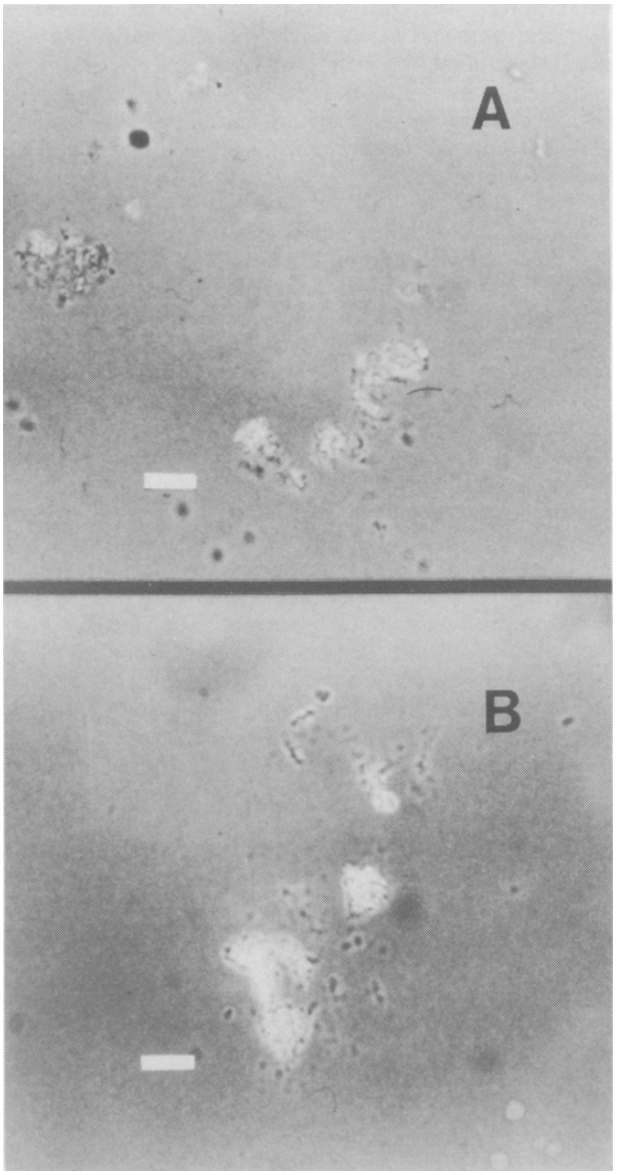

FIG. 1. Optical characteristics of (A) liposomes containing FITC-BSA and (B) egg yolk particles containing FITC-BSA, as viewed with fluorescent microscopy. Bar equals 10 μm.

TABLE 1

Moisture and Lipid Content of Oysters[a]

	Tissue		
	Total oyster	Stomach	Gill, mantle, muscle[b]
Moisture (%)	81.9	80.3	81.7
Lipid (% of wet wt)	1.6	2.7	1.4
Triacylglycerols (% of total lipid)	9.2	16.5	7.8
Polar lipids (% of total lipid)	68.0	55.1	71.4

[a]Each value is an average of two groups with 12 oysters in each group.

[b]Tissues were pooled before analysis.

oysters was poor. On the other hand, species differences may have been responsible for the altered lipid distribution.

The phospholipid distribution in lipids of oyster tissues is shown in Table 2. The major phospholipids were choline phospholipids (CP) and ethanolamine phospholipids (EP) followed by serine phospholipids (SP). The percentage of PPL in the total oyster was less than the PPL content measured for *C. virginica* (19). The findings by Swift suggested that the PPL content in tissues would increase when oysters were starved. If this were the case for *C. gigas*, the data indicated that these oysters were not starved.

The major fatty acids found in the lipid of oyster tissue is shown in Table 3. Compared to the fatty acid composition found for *C. gigas* by Langdon and Waldock (20), the data agreed fairly well for C16 and C22 fatty acids. The large amounts of C18 fatty acids found in this study may be due to the high amounts of these acids in egg yolk (21). Oysters had been maintained on an egg yolk diet for one month. After this, the triacylglycerol components would have been modified. The fatty acid composition of this lipid has been shown to reflect that of the diet (20).

TABLE 2

Distribution of Phospholipids in Oysters

	Tissue		
	Total oyster[a]	Stomach[a]	Gill, mantle, muscle[a,b]
Inositol phospholipids	2.8	3.2	2.9
Serine phospholipids	14.3	14.1	14.2
Sphingomyelin	6.2	4.8	6.5
Phosphonolipid	15.4	13.7	15.8
Choline phospholipids	29.4	32.9	28.4
Ethanolamine phospholipids	30.0	29.0	30.4
Cardiolipin	1.9	2.3	1.8

[a]Percentage of total phospholipids.

[b]Tissues were pooled before analysis.

TABLE 3

Major Constituent Fatty Acids of the Total Lipid From Juvenile Pacific Oyster *(C. gigas)*

Fatty acid[a]	Wt %
16:0	20.40
16:1	3.12
18:0	14.79
18:1	26.50
18:2	4.26
18:3	10.96
20:4	6.49
20:5	3.23
22:4	0.89
22:5	1.17
22:6	8.19

[a]Fatty acid identified by number of carbon atoms and double bonds.

Incorporation of radioactivity from egg yolk particles. Lipid from egg yolk particles prepared with ^{14}C-PAPC was subjected to two-dimensional TLC to establish the distribution of the radiolabeled lipid into egg yolk particles. More than 97% of the radioactivity was found in CP, indicating very little degradation of the radiolabel during the preparation of particles.

Oysters killed after one day recovered 12.7% of the dpm from the radiolabeled food compared to a recovery of 22.8% by oysters fed ^{14}C-DPPC-containing liposomes for one day (9). A slightly greater recovery of radiolabel was shown after three days when oysters were fed the ^{14}C-PAPC containing egg yolk particles (12.8%) than when they had been fed di[1-^{14}C] palmitoyl phosphatidylcholine (^{14}C-DPPC)-containing liposomes (10.6%). Oysters from day 8 were found to contain 16% of the exposed dose.

Seventy-seven percent of the label fed to oysters was unaccounted for in oyster tissue or the culture vessel water. This unaccounted label could have been in expired $^{14}CO_2$ or in settled matter. Langdon and Siegfried (22) reported that aeration was not satisfactory always as a means of agitation because the turbulence produced tended to be localized around the bubble source, and food settlement occurred in less disturbed areas of the culture vessel. In addition, particle clumping would have led to increased amounts of label in settled matter. Before feeding, egg yolk particles were found to be 5 to 30 μm in diameter. While these particle diameters were capable of being filtered and ingested, clumping of particles may have prevented their ingestion. The production of bacterial films would have increased particle clumping and adherence of particles to surfaces (23).

The radiolabel was distributed in approximately equal portions between the stomach tissue and that of the gill, mantle and muscle tissue. Transport of the radiolabel from the stomach to the other tissues probably occurred via the blood. Allen and Conley (6) found that after ingestion of 1-^{14}C-palmitate, the specific activities of plasma and hemocyte lipids rose over a period of 10 hr and then maintained roughly steady levels. In addition, they found radioactivity in several lipid classes having only blood contact with the digestive tract, thus supporting the premise of transport of lipid through the hemolymph.

Metabolism of radiolabeled phosphatidylcholine. The distribution of radioactivity after lipid extraction of oyster tissue is shown in Table 4. The majority of radioactivity was found in the lipid phase. However, recovery of radioactivity in the aqueous and insoluble phase implied that metabolism of the ^{14}C-PAPC had occurred, and the metabolites were shunted into non-lipid components.

TABLE 4

Distribution of Label in Various Tissues from Oysters Fed Egg Yolk Particles Containing Phosphatidylcholine, L-α-1-Palmitoyl-2-Arachidonyl [Arachidonyl-1-^{14}C][a]

	% of total dpm in tissue			% of total dpm in tissue		
	Day 1	Day 3	Day 8	Day 1	Day 3	Day 8
	Stomach			Gill, mantle, muscle[b]		
Lipid phase	96.1	94.6	95.2	85.9	82.7	87.9
Aqueous phase	1.2	2.1	1.3	3.0	3.2	3.3
Insoluble phase	2.7	3.3	3.5	11.1	14.1	8.8

[a]Data from 12 oysters per group, two groups analyzed.

[b]Tissues were pooled before analysis.

TABLE 5

Specific Activity of Lipids Isolated from Tissues of Oysters Fed Egg Yolk Particles Containing Phosphatidylcholine, L-α-1-Palmitoyl-2-Arachidonyl [Arachidonyl-1-^{14}C]

	Stomach			Gill, mantle, muscle[a]		
	Day 1 (dpm/μg)	Day 3 (dpm/μg)	Day 8 (dpm/μg)	Day 1 (dpm/μg)	Day 3 (dpm/μg)	Day 8 (dpm/μg)
Inositol phospholipids	12.3 (1.3)[b]	24.1 (0.9)	16.0 (0.7)	3.2 (1.4)	5.1 (0.8)	8.8 (1.1)
Serine phospholipids	43.8 (20.7)	120.7 (19.8)	115.3 (22.8)	9.5 (19.4)	26.2 (19.8)	41.6 (24.2)
Sphingomyelin	2.4 (0.4)	6.0 (0.3)	5.6 (0.4)	0.5 (0.5)	1.2 (0.4)	2.3 (0.6)
Phosphonolipid	1.3 (0.6)	2.9 (0.5)	2.0 (0.4)	0.3 (0.7)	0.6 (0.5)	1.0 (0.6)
Choline phospholipids	24.4 (27.1)	83.5 (32.2)	46.4 (21.6)	11.6 (47.3)	32.8 (49.6)	35.0 (40.9)
Ethanolamine phospholipids	22.6 (22.1)	68.3 (23.2)	61.7 (25.2)	3.6 (15.7)	9.7 (15.7)	14.6 (18.3)
Cardiolipin	21.7 (1.7)	48.7 (1.3)	55.2 (1.8)	16.6 (4.3)	51.2 (4.9)	79.8 (5.9)
Triacylglycerol	27.0 (25.1)	72.8 (20.8)	68.6 (26.0)	3.9 (8.2)	8.8 (5.8)	11.3 (5.8)

[a]Tissues from gill, mantle and muscle were pooled before analysis.

[b]Numbers in parentheses represent the percentage of radioactivity from total lipid extract present in the individual lipids.

TABLE 6

Distribution of Label Between the Hexane and Water Soluble Fractions after Transesterification of Lipids from Various Tissues of Oysters Fed Egg Yolk Particles Containing Phosphatidylcholine, L-α-1-Palmitoyl-2-Arachidonyl [Arachidonyl-1-^{14}C][a]

	% of total dpm in transesterified lipid			% of total dpm in transesterified lipid		
	Day 1	Day 3	Day 8	Day 1	Day 3	Day 8
	Stomach			Gill, mantle, muscle[b]		
Hexane-soluble	61.1	nd[a]	76.3	47.6	51.1	54.4
Water-soluble	38.9	nd	23.7	52.4	48.9	45.6

[a]Not determined.

[b]Tissues were pooled before analysis.

TABLE 7

Constituent Fatty Acids (Weight %) of Various Lipids[a] from the Stomach Tissue of Juvenile Pacific Oyster *(C. gigas)*

Fatty acid[b]	EP	CP	SP	IP	CL	PPL	SPH	NL
14:0	—	—	—	11.50	1.24	6.95	18.10	2.02
16:0	9.62	22.75	3.61	17.27	12.68	46.34	16.01	9.81
16:1	2.49	3.30	—	—	—	6.00	—	—
17:0	—	—	—	—	—	2.09	—	—
18:0	18.25	8.12	23.55	29.39	15.73	24.35	25.95	12.20
18:1	18.01	38.82	12.43	25.65	34.61	11.78	28.86	53.16
18:2	3.81	7.84	1.54	3.03	2.13	1.25	5.46	10.41
18:3	7.24	2.68	27.43	6.91	4.02	—	2.24	3.23
20:4	6.17	5.66	16.08	3.59	10.99	1.25	3.37	3.89
20:5	9.36	4.55	9.63	—	4.30	—	—	3.04
22:4	2.39	—	—	2.65	—	—	—	—
22:5ω6	3.33	—	1.16	—	—	—	—	—
22:5ω3	2.69	—	—	—	—	—	—	—
22:6	16.64	6.27	4.58	—	14.31	—	—	2.25
Sum polyunsaturated	51.63	27.00	60.42	16.18	35.75	2.50	11.07	22.82

[a]EP, ethanolamine phospholipids; CP, choline phospholipids; SP, serine phospholipids; IP, inositol phospholipids; CL, cardiolipin; PPL, phosphonolipid; SPH, sphingomyelin; NL, neutral lipids.

[b]Fatty acid identified by number of carbon atoms and double bonds. Tissues were analyzed on day 0.

TABLE 8

Constituent Fatty Acids (Weight %) of Various Lipids[a] from the Gill, Mantle and Muscle Tissue[b] of Juvenile Pacific Oyster *(C. gigas)*

Fatty acid[c]	EP	CP	SP	IP	CL	PPL	SPH	NL
14:0	—	—	—	13.74	13.20	3.88	17.81	5.24
16:0	6.78	19.80	3.80	7.31	12.91	68.56	13.55	14.42
16:1	2.80	3.91	1.06	—	1.38	6.81	—	2.52
17:0	—	—	—	—	—	2.35	—	—
18:0	18.28	6.21	28.22	30.01	16.60	12.13	32.05	19.90
18:1	16.79	30.78	10.08	23.59	25.48	6.28	27.93	33.29
18:2	2.75	7.92	—	2.08	4.22	—	2.87	7.24
18:3	12.49	4.15	34.99	20.97	—	—	4.57	5.33
20:4	5.49	7.76	13.32	2.29	—	—	1.22	6.75
20:5	5.02	9.20	6.36	—	—	—	—	2.18
22:4	2.36	—	—	—	1.56	—	—	—
22:5ω6	4.12	—	—	—	—	—	—	—
22:5ω3	2.14	—	—	—	1.13	—	—	—
22:6	20.97	10.26	2.18	—	23.52	—	—	3.13
Sum polyunsaturated	55.34	39.29	56.85	25.34	30.43	0.00	8.66	24.63

[a]EP, ethanolamine; CP, choline phospholipids; SP, serine phospholipids; IP, inositol phospholipids; CL, cardiolipins; PPL, phosphonolipid; SPH, sphingmyelin; NL, neutral lipids.

[b]Tissues were pooled before analysis on day 0.

[c]Fatty acid identified by number of carbon atoms and double bonds.

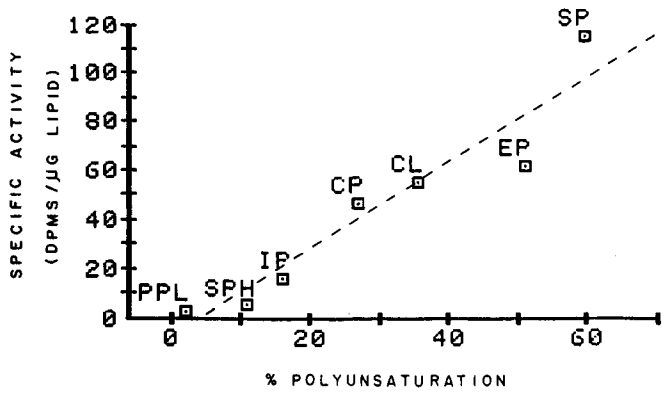

FIG. 2. Relationship between polyunsaturated fatty acids (%) and the specific activity in individual phospholipids.

This type of activity appeared to have occurred to a much greater extent in the gill, mantle and muscle tissue than in the stomach tissue.

Analysis of lipids from radiolabeled oyster tissue. After separation by two-dimensional TLC, the distribution of radioactivity among the phospholipids was determined (Table 5). The results were first expressed in terms of specific activity, dpm/μg lipid. The lipid in stomach tissue having the highest selectivity for the radiolabel was SP, followed by triacylglycerol, EP and cardiolipin (CL). The higher specific activity of SP than CP after eight days may reflect the smaller pool size of SP. In gill, mantle and muscle tissue, CL was shown to have the highest selectivity for the radiolabel, followed by CP for days 1 and 3.

By considering the total amount of radiolabel present in the lipids of each tissue, it was shown that after one day only 27% of the radiolabel remained in stomach CP. SP, EP and triacylglycerol were the other lipids in stomach tissue that had a major portion of the radiolabel. In gill, mantle and muscle tissue, 40–50% of the radiolabel remained in CP while EP and SP also had a large percentage of the radioacativity.

Analysis of radiolabeled transesterified lipid. To provide information about the routes by which radioactivity was incorporated into oyster lipids, the total lipid in each tissue was examined for intramolecular distribution of the label. In the stomach tissue, 60–75% of the radiolabel was found in the hexane soluble portion or fatty acid moiety (Table 6) compared to 100% found when oysters were fed ^{14}C-DPPC-containing liposomes (9). Transacylation could have been the main process used to transfer the radiolabeled fatty acid from CP to the other lipids in stomach tissue.

As the radiolabel was originally in arachidonic acid (AA), it was possible that the acyltransferases were acting to distribute this fatty acid for conversion into prostaglandins. Substances resembling prostaglandins have been found in scallops' tissues (24). In addition, binding sites for prostaglandins have been observed in the gill tissue of the bivalve *Modiolus demissus* (25). Therefore, one could hypothesize that the specific activity of a lipid would reflect its content of AA and its ability to serve as a precursor for prostaglandins. In this study, a correlation coefficient of 0.935 was found between the content of AA in the phospholipids and the specific activities of the individual lipids (Table 7).

An even higher correlation coefficient ($r = 0.953$) was determined for the polyunsaturated content and the specific activity of the phospholipids in stomach tissue from oysters analyzed after eight days (Figure 2). It is suggested that the fluidity of the tissue membranes regulated the activity of the acyltransferases in their transfer of the radiolabeled fatty acid (26).

For the gill, mantle and muscle tissue, 47 to 54% of the radioactivity was found in the fatty acid moiety (Table 6). Since a large portion of the radioactivity was found in the aqueous phase, in which the glycerol backbone partitions, it was not possible to correlate the specific activities observed in these tissues with the AA or polyunsaturated fatty acid content of the lipid (Table 8).

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REFERENCES

1. Swift, M.L., White, D., and Ghassemieh, M.B. (1980) *Lipids 15,* 129–132.
2. Waldock, M.J., and Holland, D.L. (1979) *Biochem. Soc. Trans. 7,* 898–900.
3. Trider, D.J., and Castell, J.D. (1980) *J. Nutr. 110,* 1303–1309.
4. Waldock, M.J., and Holland, D.L. (1984) *Lipids 19,* 332–336.
5. Joseph, J.D. (1982) *Prog. Lipid Res. 21,* 109–153.
6. Allen, W.V., and Conley, H. (1982) *Comp. Biochem. Physiol. 71B,* 201–207.
7. Lubet, P., Brichon, G., Besnard, J.Y., and Zwingelstein, G. (1985) *Comp. Biochem. Physiol. 82B,* 425–431.
8. de Moreno, J.E.A., Moreno, V.J., and Brenner, R.R. (1976) *Lipids 11,* 561–566.
9. Parker, R.S., and Selivonchick, D.P. (1986) *Aquaculture 53,* 215–228.
10. Folch, J., Lees, M., and Stanley, G.H.S. (1957) *J. Biol. Chem. 226,* 497–509.
11. Bray, G.A. (1960) *Anal. Biochem. 1,* 279–285.
12. Stillway, L.W., and Harmon, S.J. (1980) *J. Lipid Res. 21,* 1141–1143.
13. Bartlett, G.R. (1959) *J. Biol. Chem. 234,* 466–468.
14. Van Handel, E., and Zilversmit, D.B. (1957) *J. Lab. Clin. Med. 50,* 152–157.
15. Selivonchick, D.P., Johnston, P.V., and Roots, B.I. (1977) *Neurochem. Res. 2,* 379–393.
16. Sweet, C., and Zull, J.E. (1970) *Biochim. Biophys. Acta 219,* 253–262.
17. Riley, R.T. (1976) *Proc. Natl. Shellfish Assn. 65,* 84–90.
18. Watanabe, T., and Ackman, R.G. (1974) *J. Fish. Res. Board Can. 31,* 403–409.
19. Swift, M.L. (1977) *Lipids 12,* 449–451.
20. Langdon, C.J., and Waldock, M.J. (1981) *J. Mar. Biol. Ass. U.K. 61,* 431–448.
21. Shenstone, F.S. (1968) in *Egg Quality. A Study of the Hen's Egg,* (Carter, T.C., ed.) pp. 26–64, Oliver & Boyd, Edinburgh.
22. Langdon, C.J., and Siegfried, C.A. (1984) *Aquaculture 39,* 135–153.
23. Masson, M. (1977) *Mar. Biol. 40,* 157–164.
24. Nomura, T., Ogata, H., and Lucas, A. (1979) *Oceanogr. Med. 53/54,* 87–94.
25. Freas, W., and Grollman, S. (1981) *J. Exp. Zool. 216,* 225–233.
26. Thompson, G.A. Jr. (1980) in *The Regulation of Membrane Lipid Metabolism,* p. 98, CRC Press, Inc., Boca Raton, FL.

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Time Course of Dolichol and Dolichyl Phosphate in Regenerating Rat Liver

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By using novel high performance liquid chromatographic methods, the time course of dolichol and dolichyl phosphate in regenerating rat liver was investigated and compared with that for cholesterol. The cholesterol level in the liver increased more rapidly than the level of dolichyl phosphate (Dol-P). HMG-CoA reductase activity correlated well with cholesterol concentration. The level of Dol-P at day 1 was lower than that in normal liver, but was increased at three to five days after hepatectomy, which correlates well with mannosyl transferase activity. Dolichol decreased for three days, and thereafter increased until 11 days after hepatectomy. The compositions of dolichol and Dol-P homologues did not change during the experimental period. The results indicate that the cholesterol content in regenerating rat liver increased first, followed by Dol-P, and finally dolichol.
Lipids 23, 28–31 (1988).

Dolichol consists of a group of polyisoprenols with 16 to 22 isoprene units. Dolichols occur widely in tissues and biological fluids as free alcohols, phosphate esters and fatty acid esters (1). In many mammals and yeasts, dolichyl phosphate (Dol-P) serves as a carrier of saccharide moieties in the transfer of sugars to asparagine residues of polypeptides (2–6). The function of free dolichol and dolichyl esters, however, has not been established. Since dolichol, cholesterol and ubiquinone are synthesized by a branched pathway in mevalonate metabolism or through the major branch-point of farnesyl pyrophosphate, many investigators have been interested in the regulation of dolichol biosynthesis. Dolichol synthesis has been studied in the developing sea urchin embryo (7), erythropoietic spleen (8) and the developing mouse brain (9).

Because the biosynthesis of glycoproteins in regenerating rat liver after partial hepatectomy is altered (10–12), it is anticipated that dolichol content of regenerating liver also may be altered. Thus, dolichol in liver, liver microsomes and plasma, and Dol-P in liver were determined in regenerating rat liver by the methods previously described. This paper presents the results and the time course of HMG CoA reductase activity, which is the major regulatory enzyme of cholesterol and dolichol biosynthesis, and mannosyl transferase activity, which may be supported by Dol-P endogenous to the enzyme preparation.

MATERIALS AND METHODS

Chemicals and reagents. Dolichol and Dol-P homologs used were the preparations described previously (13). Didecaprenylethanol used as an internal standard for dolichol and 2,2-didecaprenylethyl phosphate as an internal standard for Dol-P were synthesized in our laboratories (13,14). Anthracene-9-carboxylic acid was purchased from Aldrich Chemical Company (Milwaukee, WI). 3-(9-Anthryl)-diazo-2-propene was prepared from 3-(9-anthryl)-propenal by the method of Nakaya et al. (15). The standard dolichol, Dol-P and internal standards were dissolved in ethyl ether and stored at −20 C. GDP-[^{14}C]mannose and DL-[3-^{14}C]HMG CoA were purchased from Amersham International (Buckinghamshire, UK). All other reagents were analytical grade.

Animals. Male Sprague-Dawley rats weighing ca. 240 g were used. Partial hepatectomy of about 70% of the liver was performed under diethyl ether anesthesia by the method of Higgins and Anderson (16), and control rats were subjected to a sham operation. The animals were killed at the appropriate time after the operation, and the regenerating livers were separated from the nonregenerated portion and stored frozen at −80 C until analysis.

Preparation of rat liver microsomes. The liver was homogenized in 0.25 M sucrose aqueous solution. The homogenates were centrifuged at 10,000 × g for 20 min, and the supernatants were centrifuged at 100,000 × g for 60 min. The microsomes thus pelleted were washed by suspension in 0.15 M Tris-HCl buffer (pH 7.9) and centrifuged at 100,000 × g for 60 min. The washed microsomes were resuspended in 0.25 M sucrose.

Determination of dolichol and Dol-P. For the measurement of dolichol, the liver (ca. 100 mg) was homogenized in 2 ml of distilled water. First, 0.25 ml of 50% KOH aqueous solution and 3 ml of 1% pyrogallol in methanol were added successively to 2 ml of the sample. This mixture was heated at 70 C for 60 min and then cooled to room temperature. The saponified mixture was extracted with n-hexane, evaporated to dryness and analyzed by HPLC with UV detection using 2,2-didecaprenylethanol as an internal standard. The rat plasma and liver microsomes were subjected to alkaline hydrolysis, extracted with n-hexane, derivatized with anthracene-9-carboxylic acid and analyzed by HPLC with fluorescence detection. For measurement of Dol-P homologs, the livers (ca. 100 mg) were homogenized in 2 ml of distilled water. First, 1 ml of 1 N HCl aqueous solution was added to the homogenate, and the mixture was allowed to stand at room temperature for 45 min. This mixture was heated at 70 C for 45 min and then cooled to room temperature. The hydrolyzed mixture was extracted with diethyl ether, derivatized with 3-(9-anthryl)-diazo-2-propene and then determined by HPLC with fluorescence detection using 2,2-didecaprenylethyl phosphate as an internal standard. The HPLC methods have been reported in detail elsewhere (13,14).

Determination of cholesterol in rat liver. Approximately 100 mg of rat liver was homogenized in 1 ml of distilled water. The homogenate was extracted twice with four volumes of chloroform/methanol (2:1). The organic phase was evaporated to dryness under N_2 gas, and the residue was dissolved in 1 ml of chloroform/methanol (2:1). A 100 μl aliquot of the solution was evaporated to dryness, and the residue was dissolved in 100 μl of isopropyl

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Abbreviation: Dol-P, dolichyl phosphate.

alcohol. Cholesterol in the isopropyl alcohol solution was measured by using Iatro Lipo TC (Iatro Inc., Tokyo, Japan), a reagent kit for the assay of cholesterol by a cholesterol oxidase method.

Assay for transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to lipid intermediates. One ml of the reaction mixture consisted of 30 mM Tris-HCl buffer (pH 7.8), 1 mM $MnCl_2$, 12.5 mM mercaptoethanol, 2 mM AMP, 0.5 μCi of GDP-[^{14}C]mannose and microsomes (1.2 mg protein). The reaction was started by adding the radioactive substrate, and incubation was performed at 30 C for 15 min. After incubation, dolichyl mannosyl phosphate was extracted with chloroform/methanol (2:1) and analyzed by thin layer chromatography (17).

Assay for HMG CoA reductase activity. The reaction mixture consisted of 200 mM phosphate buffer (pH 7.4), 100 mM EDTA, 100 mM dithiothreitol, 50 mM NADPH, 1 mM DL-[3-^{14}C]HMG CoA, and microsomes (0.12 mg protein). The reaction was started by addition of microsomes and continued at 37 C for 20 min. The reaction was terminated by adding 10 μl of 2 N HCl, and the resulting mixture was further incubated at 37 C for 15 min. Then, the mixture was analyzed by thin layer chromatography (18).

RESULTS AND DISCUSSION

Time course of liver weight after partial hepatectomy. Wet weight of rat liver after partial hepatectomy was measured during the first 20 days, and the regenerative ratio of the liver was calculated (16) (Fig. 1). Regeneration proceeded rapidly during the first five days, reached 90% by day 11 and was completed by day 13.

Concentration of dolichol and Dol-P in regenerating liver. Dolichol and Dol-P in regenerating liver were analyzed by HPLC; the results are shown in Figure 2. The concentration of dolichol in the liver decreased gradually during the first three days, and at day 3 was significantly lower than that of normal liver, 13.6 + 1.34 μg/g vs 21.2 + 1.01 μg/g. From day 3, the concentration of dolichol increased gradually, and on day 11 reached a peak that was 1.5 times higher than that in normal liver. The dolichol concentration of normal liver described here agrees with the data of Keller et al. (19) and Eggens et al. (20). However, the time course of Dol-P was different from that of dolichol. The Dol-P level on day 1 was lower than that of the normal rat liver but increased rapidly during the next three days. The Dol-P level on day 5 was significantly higher than that of normal liver, 5.2 + 0.32 μg/g vs 3.0 + 0.22 μg/g. On the other hand, the concentration of dolichol and Dol-P in the control liver did not change at all in the course of the experiments. The observed normal liver level of Dol-P is in good agreement with the data of Chaudhary et al. (21). Keller et al. (19) have reported the total Dol-P (free and chemically bound Dol-P) levels in rat liver but their values are eight times higher than the control Dol-P levels presented here. This difference may be attributable to the different hydrolysis methods used. We confirmed that our assay could determine the concentrations of free Dol-P and Dol-P monosaccharides in rat liver. These results indicate that the synthesis of Dol-P might precede that of dolichol in the regenerating liver.

Figure 3 shows the mannosyl transferase activity in the regeneraing liver microsomes. The activity was lower on day 1 but was significantly higher on days 3 and 5 than that of the normal liver microsomes. The time course of mannosyl transferase activity coincided with that of Dol-P level in the regenerating liver (Fig. 2). This result suggests that Dol-P levels may regulate mannosyl transferase activity. Figure 4 shows chromatograms of dolichol and Dol-P in the regenerating rat liver on day 5. As can be seen from the chromatograms, changes of the composition of dolichol or of Dol-P were observed during the experimental period.

Concentration of dolichol in microsomes and plasma. Figure 5 shows the time course of the dolichol concentration in the liver microsomes and plasma of partially hepatectomized rats. The time course of the concentration of microsomal dolichol was in good agreement with

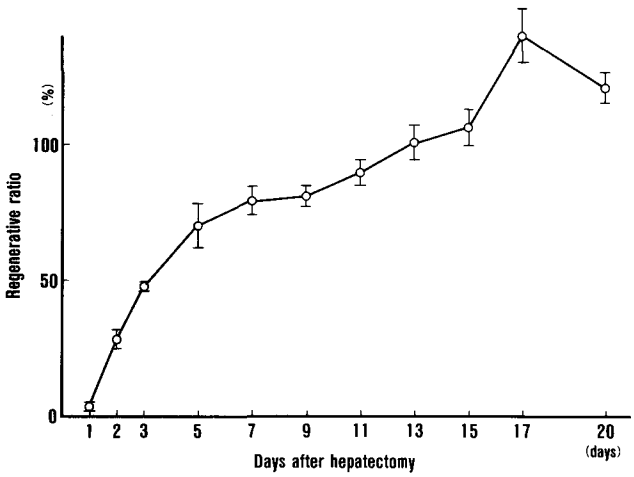

FIG. 1. Time course of liver weight after partial hepatectomy in terms of the regenerative ratio of the liver, calculated by the method of Higgins and Anderson (16). Vertical bars denote SE of the means of three separate experiments.

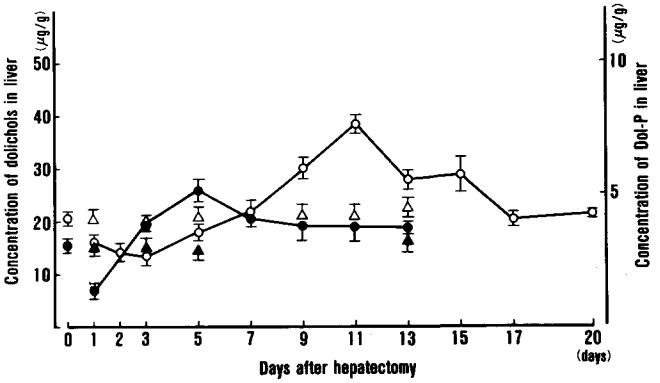

FIG. 2. Time course of the concentrations of dolichol and Dol-P in the regenerating liver. The concentration of dolichol (—○—) and Dol-P (— ● —) in the regenerating liver and the control value of dolichols (—△—) and Dol-P (—▲—) were analyzed as described in Materials and Methods. Vertical bars denote SE of the means of three separate experiments.

that of liver dolichol (Fig. 2). In contrast, the plasma level of dolichol did not decrease but was increased on day 3 when the liver dolichol concentration was lowest during the experimental period. The concentration of microsomal and plasma dolichol in the control did not change at all during the course of experiments. This result suggests that dolichol in plasma might not have originated from the liver. However, it is possible that plasma dolichol originated from liver since dolichol might be released to the plasma as a result of the hepatectomy.

Concentration of total cholesterol and HMG CoA reductase activity in regenerating liver. Figure 6 shows the time course of total cholesterol in the regenerating rat liver and that of HMG CoA reductase activity in the liver microsomes. The cholesterol level in regenerating liver was increased one and three days after hepatectomy,

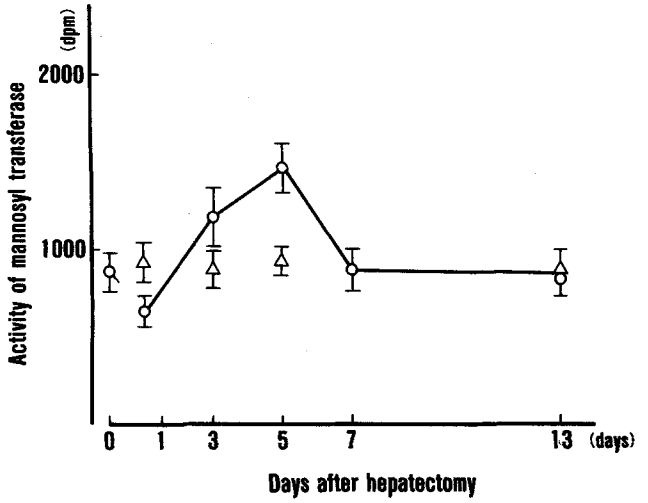

FIG. 3. Time course of mannosyl transferase activity in the regenerating liver microsomes (—○—) and the control activity (—△—). The assay condition were as described under Materials and Methods. Vertical bars denote SE of the means of three separate experiments.

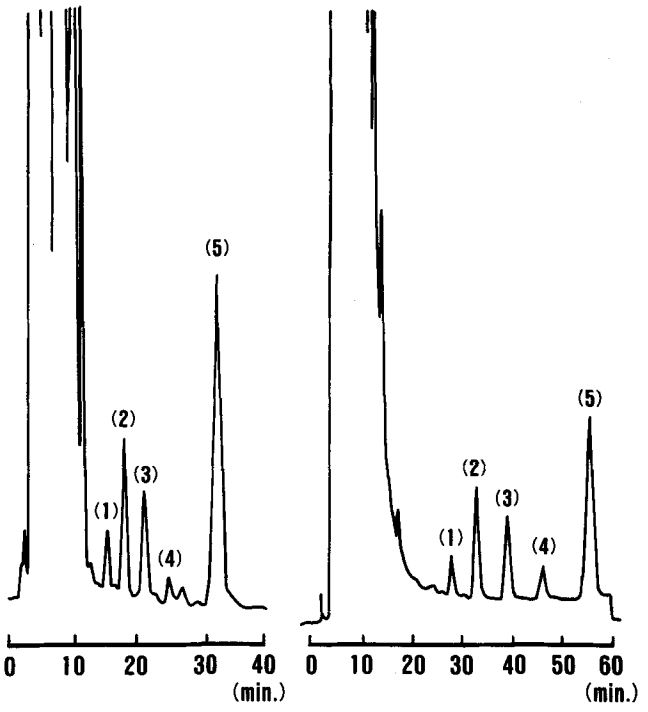

FIG. 4. Chromatograms of dolichol (left) and Dol-P (right) in the regenerating rat liver on day 5. Dolichol and Dol-P were extracted as described in Materials and Methods. Peaks were identified as follows: left: (1) dolichol-17; (2) dolichol-18; (3) dolichol-19; (4) dolichol-20; (5) 2,2-didecaprenyl ethanol (internal standard); right: (1) Dol-P-17; (2) Dol-P-18; (3) Dol-P-19; (4) Dol-P-20; (5) 2,2-didecaprenylethyl phosphate (internal standard).

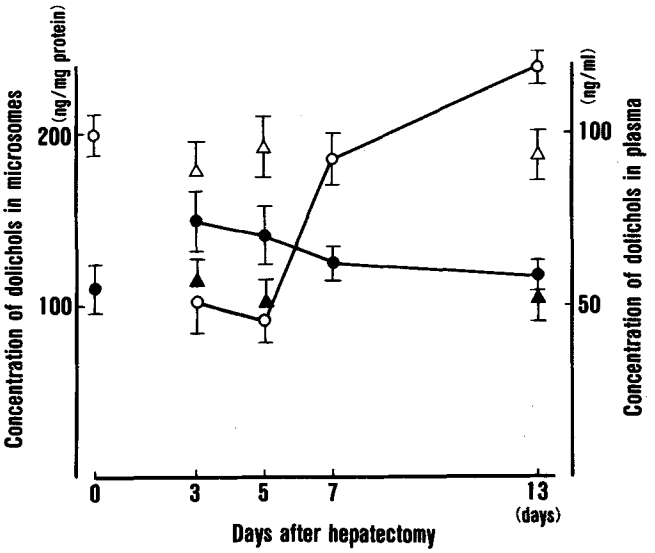

FIG. 5. Time course of the concentration of dolichol in regenerating liver microsomes (—○—) and plasma (— ● —) and the control value of dolichol in microsomes (—△—) and plasma (—▲—). Rat liver microsomes (1 mg protein) or plasma (1 ml) was extracted as described in Materials and Methods. Vertical bars denote SE of the means of three separate experiments.

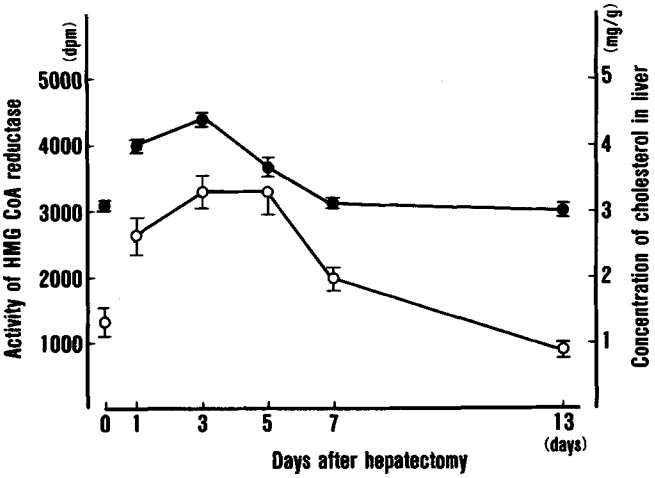

FIG. 6. Time course of the concentration of total cholesterol (— ● —) and HMG CoA reductase activity (—○—) in regenerating liver. The assay conditions were as described under Materials and Methods. Vertical bars denote SE of the means of three separate experiments.

reaching a maximum of 1.5 times that of normal liver, 3.10 + 0.09 mg/g. Subsequently, the level rapidly returned to normal. The time course of HMG CoA reductase activity in the regenerating liver microsomes was similar to that of the total cholesterol in the regenerating liver. The results clearly indicate that in the regenerating liver cholesterol increases first, followed by Dol-P and then dolichol.

James and Kandutsch (20) and Sakakihara and Volpe (9) have studied the regulation of dolichol and cholesterol synthesis in developing brain, and they found that the synthesis of cholesterol preceded that of dolichol. Their observations are in good agreement with our results in regenerating rat liver but they did not examine the synthesis of Dol-P. Since Dol-P serves as the carrier of saccharide moieties in the synthesis of asparagine-linked glycoproteins, the synthesis of Dol-P is quite important (23-25). Marino et al. (12) have recently studied the synthesis of dolichol and Dol-P in the earlier stage (16 hr after partial hepatectomy) of rat liver regeneration. These authors showed that the dolichol content and its synthesis from mevalonate were increased but no variation in Dol-P was observed. The data obtained for the dolichol and Dol-P concentrations on day 1 were different. This may be attributable to somewhat different experimental conditions. We believe that our results have clarified the relationship between dolichol and Dol-P in the regenerating liver, and our data suggest that the synthesis of Dol-P precedes the synthesis of dolichol.

REFERENCES

1. Butterworth, P.H.W., and Hemming, F.W. (1968) *Arch. Biochem. Biophys. 128,* 503-508.
2. Gough, D.P., and Hemming, F.W. (1970) *Biochem. J. 118,* 163-166.
3. Grange, D.K., and Adair, W.L. (1977) *Biochem. Biophys. Res. Commun. 79,* 734-740.
4. Lucas, J.J., Waechter, C.J., and Lennarz, W.J. (1975) *J. Biol. Chem. 250,* 1911-2002.
5. Waechter, C.J., and Lennarz, W.J. (1976) *Annu. Rev. Biochem. 45,* 95-112.
6. Parodi, A.J., and Leloir, L.F. (1979) *Biochim. Biophys. Acta 559,* 1-37.
7. Carson, D.C., and Lennarz, W.J. (1981) *J. Biol. Chem. 256,* 4679-4686.
8. Potter, J.E.R., James, M.J., and Kandutsch, A.A. (1981) *J. Biol. Chem. 256,* 2371-2376.
9. Sakakihara, Y., and Volpe, J.J. (1984) *Develop. Brain Res. 14,* 255-262.
10. Okamoto, Y., and Akamatsu, N. (1977) *Biochim. Biophys. Acta 498,* 272-281.
11. Okamoto, Y., and Akamatsu, N. (1980) *Biochem. J. 188,* 905-911.
12. Marino, M., Bruscalupi, G., Spagnuolo, S., Leoni, S., Mangiantini, M.T., Trentalance, A., and Hemming, F.W. (1986) *Biosci. Rep. 6,* 409-413.
13. Yamada, K., Yokohama, H., Abe, S., Katayama, K., and Sato, T. (1985) *Anal. Biochem. 150,* 26-31.
14. Yamada, K., Abe, S., Katayama, K., and Sato, T. (1986) *Anal. Biochem. 156,* 380-385.
15. Nakaya, T., Tomomoto, T., and Imoto, M. (1967) *Bull. Chem. Soc. Jpn. 40,* 691-692.
16. Higgins, G.M., and Anderson, R.M. (1931) *Arch. Pathol. 12,* 186-202.
17. Eggens, I., Eriksson, L.C., Chojnacki, T., and Dallner, G. (1984) *Cancer Res. 44,* 799-805.
18. Kuroda, M., Tanzawa, K., Tsujita, Y., and Endo, A. (1977) *Biochim. Biophys. Acta 489,* 119-125.
19. Keller, R.K., Fuller, M.S., Rottler, G.D., and Connelly, L.W. (1985) *Anal. Biochem. 147,* 166-173.
20. Eggens, I., Chojnacki, T., Kenne, L., and Dallner, G. (1983) *Biochim. Biophys. Acta 751,* 355-368.
21. Chaudhary, N., Freeman, D.J., Rip, J.W., and Carroll, K.K. (1982) *Lipids 17,* 558-560.
22. James, M.J., and Kandutsch, A.A. (1980) *Biochim. Biophys. Acta 619,* 432-435.
23. Rossignol, D.P., Scher, M., Waechter, G.J., and Lennarz, W.J. (1983) *J. Biol. Chem. 258,* 9122-9127.
24. Ekstrom, T.J., Chojnacki, T., and Dallner, G. (1984) *J. Biol. Chem. 259,* 10460-10468.
25. Keller, R.K. (1986) *J. Biol. Chem. 261,* 12053-12059.

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Lysophosphatidylcholine Accumulation in the Ischemic Canine Heart

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The production of cardiac arrhythmias and the elevation of lysophosphatidylcholine level in the ischemic myocardium have been well-documented in a number of studies. However, the relationship between the production arrhythmias and the elevation of tissue lysophosphatidylcholine level was not reported. In this study, the lysophosphatidylcholine level and the occurrence of cardiac arrhythmias in the ischemic canine heart were monitored. A temporal relationship between the accumulation of lysophosphatidylcholine and the occurrence of arrhythmias was established after five hr of ischemia. A significant elevation of lysophosphatidylcholine was detected at three hr of ischemia without the occurrence of arrhythmias. The results indicate that cardiac arrhythmias did not cause the elevation of lysophosphatidylcholine and if lysophospholipids are causally related to the arrhythmias that a critical level of the lysophospholipid must accumulate in order to elicit electrophysiological alterations.
Lipids 23, 32-35 (1988).

The development of ventricular arrhythmias after the onset of myocardial ischemia is well-documented (1,2). However, the exact biochemical cause for the production of arrhythmias remains largely unknown. In the last decade, the accumulation of lysophospholipids in the ischemic myocardium has been suggested as one of the biochemical factors in the generation of arrhythmias (3,4). Exogenous addition of lysophospholipids have been shown to cause the depression of action potential in isolated cardiac fibers (5) and to decrease the membrane excitability in Purkinje fibers (6). Higher concentrations of lysophospholipids in the superfusate were found to produce inexcitability and sustained rhythmic activity (6). Addition of lysophosphatidylcholine in the perfusate was shown to cause the production of cardiac arrhythmias in the isolated heart (7,8).

Although the ability of exogenous lysophospholipid to cause arrhythmias is irrefutable, a direct relationship between the development of arrhythmias and the accumulation of lysophospholipids in the ischemic myocardium had not been established. In addition, there had been difficulties in the quantitation of lysophospholipids in the heart.

Lysophosphatidylcholine is the major lysophospholipid in the mammalian heart. We reported in our earlier study that there was an accumulation of lysophosphatidylcholine in the canine myocardium after 24 hr of coronary artery occlusion (9). Since arrhythmias are produced by this model after different periods of ischemia, the objective of this study was to examine the relationship between lysophosphatidylcholine accumulation and the occurrence of cardiac arrhythmias.

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Abbreviation: PVC's, premature ventricular contractions.

MATERIALS AND METHODS

Materials. Lysophosphatidylcholine prepared from egg yolk was obtained from Sigma Chemical Co. (St. Louis, MO). Phosphatidylcholine and lysophosphatidylcholine standards were products of Serdary Research Labs. (London, Ontario, Canada). Thin layer chromatographic plates (SIL-G25) were obtained from Brinkmann (Rexdale, Ontario, Canada). All other chemicals were of reagent grade and were obtained from Fisher Chemical Co. (Edmonton, Alberta, Canada).

Methods. Mongrel dogs of either sex, weighing 8-15 kg, were used for this study. The dogs were anesthetized by IV injection of sodium pentobarbital (30 mg/kg). Respiration was maintained by a Harvard pump. The lead II electrocardiogram and blood pressure were monitored continuously throughout the experiment. The heart was exposed by a left thoracotomy. The left anterior descending coronary artery was isolated at a site proximal to the first branch (1-1.5 cm from the origin of the artery). The artery was occluded by the Harris two-stage procedure (10). A total of 34 dogs underwent the occlusion procedure. Four dogs developed ventricular fibrillation and subsequently died (2 in the 1-hr group and 1 each in the 3 and 5-hr group). In the remaining animals, hearts were removed at the end of 1, 3, 5, 8 and 12 hr of ischemia (7, 6, 6, 6 and 5 animals in each group respectively). Tissue samples were taken from the ischemic and nonischemic areas of the left ventricle as described previously (9). After weighing, the samples were used immediately for lipid extraction.

The electrocardiograms were later analyzed over a period of three to five min. The average number of premature ventricular contractions (PVC's) per minute was determined for comparison over the various time periods of ischemia. The number of PVC's/min was used as a measure of the frequency of cardiac arrhythmias.

Lipid analysis. Lipids were extracted from the tissue using a neutral solvent system (11). The tissue was homogenized in 10 vol (w/v) of chloroform/methanol (1:2, v/v) and extracted twice with the same solvent. The extracts were pooled and a biphasic mixture was obtained by the addition of 0.1 M KCl to provide a solvent mixture of $CHCl_3/CH_3OH/H_2O$ (2:1:0.8, v/v/v). The lower phase was evaporated under reduced pressure. The lipid extract was reconstituted in a small volume of chloroform/methanol (2:1) and was used immediately for lipid analysis. The phospholipids in the lipid extract were separated by thin layer chromatography in a solvent containing chloroform/methanol/water/acetic acid (75:60:8:10, v/v/v/v) (11). The phospholipid fractions on the thin layer chromatographic plates were visualized by iodine vapor and identified by phospholipid standards. Quantitation of lipid phosphorus was performed by the method of Bartlett (12).

The dry wt of the tissue was determined by complete dehydration in an oven at 100 C under reduced pressure. Total phospholipid content was assessed by the method

of Raheja (13). Quantitation of total cholesterol was performed by enzymatic assay with cholesterol oxidase (14).

Statistical analysis. Values were expressed as mean ± standard deviation. Analysis of variance and Student's t-test for paired or unpaired data were used where appropriate. A p-value <0.05 was considered significant.

RESULTS

The alterations of lysophosphatidylcholine concentration in the ischemic and non-ischemic areas of canine hearts after 1, 3, 5, 8 and 12 hr of coronary artery occlusion are summarized in Table 1. The results were expressed as dry wt instead of wet wt since edema, as indicated by a significant increase in tissue water in the ischemic area, was evident after 8 and 12 hr of coronary artery occlusion (Table 2). Hence, all subsequent data were calculated as per g dry wt to correct for the increase in tissue water after ischemia. There was no significant change in lysophosphatidylcholine content after one hr of ischemia. The increase in lysophosphatidylcholine in the ischemic areas as compared to the nonischemic areas of the same hearts (9) was significant after three hr of ischemia (Table 1). The percentage increases in lysophosphatidylcholine content of the ischemic area compared with the nonischemic area after 3, 5, 8 and 12 hr of ischemia were 28, 53, 104 and 171% respectively. At 12 hr of coronary artery occlusion, the increase in lysophosphatidylcholine in the ischemic area was significantly higher than the increases observed at one, three, five or eight hr.

Cardiac arrhythmias occurred during the initial phase after the occlusion of the coronary artery. The arrhythmias usually subsided over the next 30 min. Subsequently, the number of PVC's/min increased with the duration of ischemia. Significant increases in PVC's/min were observed after 8 and 12 hr of coronary artery occlusion. The relationship between the percentage increase of lysophosphatidylcholine in the ischemic area and the occurrence of PVC is depicted in Figure 1. It is evident that the time course of lysophosphatidylcholine accumulation appears to precede the increase in the number of PVC's/min.

The total phospholipid, phosphatidylcholine and total cholesterol in the nonischemic and ischemic areas after various time periods are presented in Table 3. There was no significant change in any of these lipids.

TABLE 1

Lysophosphatidylcholine Content in Nonischemic and Ischemic Regions of Canine Hearts

		Lysophosphatidylcholine concentration μmol/g dry wt	
1 hr	Nonischemic	0.633 ± 0.365	n = 7
	Ischemic	0.614 ± 0.393	
3 hr	Nonischemic	0.732 ± 0.347	n = 6[a]
	Ischemic	0.937 ± 0.385	
5 hr	Nonischemic	0.458 ± 0.143	n = 6[b]
	Ischemic	0.702 ± 0.184	
8 hr	Nonischemic	0.467 ± 0.097	n = 6[a]
	Ischemic	0.953 ± 0.218	
12 hr	Nonischemic	0.553 ± 0.143	n = 5[b]
	Ischemic	1.500 ± 0.467	

Values represent mean ± standard deviation; n, number of experiments.
[a]p < 0.01.
[b]p < 0.05.

DISCUSSION

The arrhythmogenic (3,4) and cytolytic (15) actions of lysophosphatidylcholine have put a definite focus on the need to quantitate this lysophospholipid in the cardiac

TABLE 2

Tissue Water in Nonischemic and Ischemic Regions of Canine Hearts

		g dry wt/g wet wt		g H_2O/g dry wt	
1 hr	Nonischemic	0.230 ± 0.008	n = 3	3.36 ± 0.15	n = 3
	Ischemic	0.224 ± 0.008		3.51 ± 0.12	
3 hr	Nonischemic	0.229 ± 0.002	n = 3	3.38 ± 0.04	n = 3
	Ischemic	0.207 ± 0.017		3.85 ± 0.38	
5 hr	Nonischemic	0.227 ± 0.002	n = 3	3.42 ± 0.04	n = 3
	Ischemic	0.210 ± 0.002		3.79 ± 0.42	
8 hr	Nonischemic	0.229 ± 0.008	n = 4[a]	3.38 ± 0.16	n = 4[b]
	Ischemic	0.189 ± 0.003		4.31 ± 0.09	
12 hr	Nonischemic	0.233 ± 0.003	n = 3[b]	3.30 ± 0.06	n = 3[b]
	Ischemic	0.202 ± 0.009		4.00 ± 0.21	

Values represent mean ± standard deviation; n, number of experiments.
[a]p < 0.01.
[b]p < 0.05.

TABLE 3

Lipid Content in Nonischemic and Ischemic Regions of Canine Hearts

		Total phospholipid (μmol phosphate/g dry wt)		Phosphatidylcholine (μmol phosphate/g dry wt)		Total cholesterol (mg/g dry wt)	
1 hr	Nonischemic	101.6 ± 15.9	n = 3	48.0 ± 3.8	n = 7	8.22 ± 1.55	n = 3
	Ischemic	113.0 ± 7.7		47.5 ± 2.9		9.04 ± 2.88	
3 hr	Nonischemic	114.4 ± 9.6	n = 3	44.7 ± 6.2	n = 6	10.04 ± 0.82	n = 3
	Ischemic	114.9 ± 3.5		47.6 ± 8.7		8.29 ± 1.13	
5 hr	Nonischemic	101.0 ± 4.3	n = 3	43.4 ± 6.6	n = 7	8.42 ± 0.89	n = 3
	Ischemic	107.2 ± 9.7		42.2 ± 4.2		9.77 ± 1.27	
8 hr	Nonischemic	102.1 ± 6.3	n = 4	52.5 ± 10.5	n = 6	9.35 ± 3.11	n = 4
	Ischemic	115.5 ± 7.6		49.1 ± 8.5		10.45 ± 1.56	
12 hr	Nonischemic	94.1 ± 4.4	n = 3	45.1 ± 2.4	n = 5	9.05 ± 2.00	n = 3
	Ischemic	94.8 ± 11.1		47.7 ± 3.5		8.00 ± 1.36	

Values represent mean ± standard deviation; n, number of experiments.

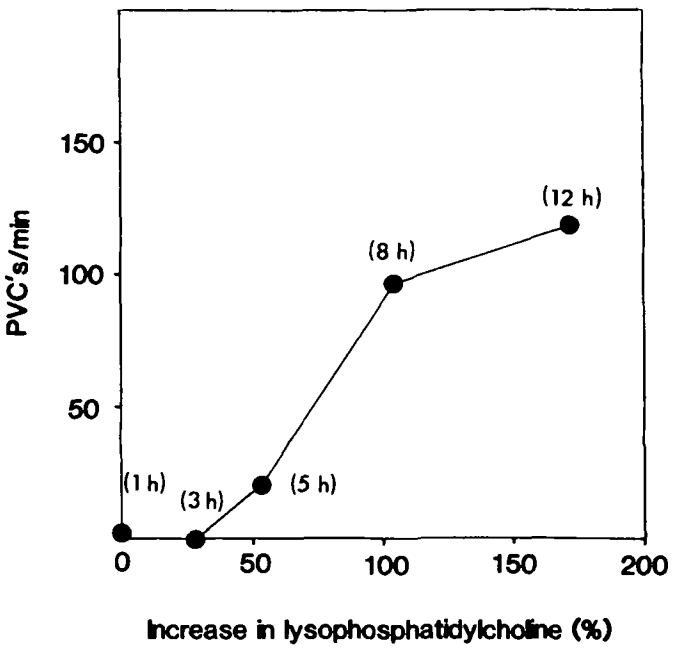

FIG. 1. The relationship between the accumulation of lysophosphatidylcholine and the frequency of cardiac arrhythmias during myocardial ischemia. Ischemia in the left ventricle of the canine heart was produced by the occlusion of the left anterior descending coronary artery. The number of premature ventricular contractions (PVC's) and the lysophosphatidylcholine content in the ischemic and nonischemic tissues were determined at different periods after the occlusion. The percentage increase of lysophosphatidylcholine in the ischemic tissue was calculated by comparison with the corresponding nonischemic tissue. The numbers in parentheses are the time points after the occlusion of the left anterior descending coronary artery.

tissue. The use of acidic extraction medium that caused the hydrolysis of phospholipid plasmalogens has been shown to produce a high lysophospholipid content (16). In this study, a neutral extraction medium that was shown to provide an accurate assessment of cellular lysophosphatidylcholine content was used (11,16). In addition, the lysophosphatidylcholine levels in the control and ischemic tissues subsequently were confirmed by a radiolabeled procedure developed in our laboratory (17).

It is clear from this study that lysophosphatidylcholine level significantly was increased after three hr of ischemia. The increase reached a maximum at 12 hr of ischemia, and no further increase was observed at 24 hr of ischemia (9). The results are expressed on the basis of the tissue dry wt since edema was apparent after prolonged period of ischemia. No significant change in total phospholipid, total cholesterol or phosphatidylcholine was detected at all time points. Although the increase in lysophosphatidylcholine probably arose from the catabolism of phosphatidylcholine, we were not able to detect such small changes in phosphatidylcholine due to its relatively large pool in comparison with lysophosphatidylcholine.

Elevated levels of lysophosphatidylcholine in the ischemic tissues were reported in a number of studies using a variety of animal models. In all cases, a significant increase in lysophosphatidylcholine levels (50% or more) was found in the ischemic myocardium (9,16,18,19). A recent report by Steenbergen and Jennings (19) noted that significant elevation of tissue lysophosphatidylcholine was not detected in the canine myocardium until after 2.5 hr using a model of in vitro ischemia. This was in contrast to previous reports that lysophosphatidylcholine level was increased in much shorter periods in porcine (16) or feline (18) hearts. A difference in species has been suggested as a reason for these apparent discrepancies (4). The result obtained from this study qualitatively and quantitatively is comparable to those reported by Steenbergen and Jennings (19). This is not surprising since canine heart was used in both studies.

The production of arrhythmias and the elevation of lysophosphatidylcholine level in the ischemic heart have been well-documented separately in various studies. However, the relationship between arrhythmia production and elevation of lysophosphatidylcholine level was not clear. It also is not known if the accumulation of

lysophosphatidylcholine may be related causally to the occurrence of cardiac arrhythmias. In this study, the monitoring of the premature ventricular contraction provided us with a facile and quantitative approach to determine the occurrence of arrhythmias. Our results clearly demonstrate a temporal relationship between the lysophosphatidylcholine level and the frequency of arrhythmias after five hr of coronary artery occlusion. It should be noted that a significant elevation of lysophosphatidylcholine level was observed at three hr of ischemia without the occurrence of arrhythmias. If lysophosphatidylcholine is a biochemical factor in the production of arrhythmias, it appears that a critical quantity of lysophosphatidylcholine (a two-fold increase) must accumulate in order to elicite electrophysiological alterations. We conclude that elevation of the lysophospholipid cannot be a consequence of cardiac arrhythmias.

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REFERENCES

1. Elharrar, V., and Zipes, D.G. (1977) *Am. J. Physiol. 233,* H329-H345.
2. Wit, A.L., and Rosen, M.R. (1983) *Am. Heart J. 106,* 829-839.
3. Katz, A.M., and Messineo, F.C. (1981) *Circ. Res. 48,* 1-16.
4. Corr, P.B., Gross, R.W., and Sobel, B.E. (1984) *Circ. Res. 55,* 135-154.
5. Corr, P.B., Cain, M.E., Witkowski, F.X., Price, D.A., and Sobel, B.E. (1979) *Circ. Res. 44,* 822-832.
6. Arnsdorf, M.F., and Sawicki, G.J. (1981) *Circ. Res. 49,* 16-30.
7. Man, R.Y.K., and Choy, P.C. (1982) *J. Mol. Cell. Cardiol. 14,* 173-175.
8. Man, R.Y.K., and Lederman, C.L. (1985) *Pharmacology 31,* 11-16.
9. Man, R.Y.K., Slater, T.L., Pelletier, M.P.J., and Choy, P.C. (1983) *Lipids 18,* 677-681.
10. Harris, A.S. (1950) *Circulation 1,* 1318-1328.
11. Mock, T., Pelletier, M.P.J., Man, R.Y.K., and Choy, P.C. (1984) *Anal. Biochem. 137,* 277-281.
12. Bartlett, G.R. (1958) *J. Biol. Chem. 234,* 466-468.
13. Raheja, R.K., Kaur, C., Singh, A., and Bhatia, I.S. (1973) *J. Lipid Res. 14,* 695-697.
14. Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W., and Fu, P.C. (1974) *Clin. Chem. 20,* 470-475.
15. Weltzien, H.U. (1979) *Biochim. Biophys. Acta 559,* 259-287.
16. Shaikh, N.A., and Downar, E. (1981) *Circ. Res. 49,* 316-325.
17. Wietzek, M., Arthur, G., Man, R.Y.K., and Choy, P.C. (1985) *J. Lipid Res. 26,* 1166-1169.
18. Corr, P.B., Snyder, D.W., Lee, B.I., Gross, R.W., Keim, C.R., and Sobel, B.E. (1982) *Am. J. Physiol. 243,* H187-H195.
19. Steenbergen, C., and Jennings, R.B. (1984) *J. Mol. Cell. Cardiol. 16,* 605-621.

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Free Fatty Acid Inhibition of the Insulin Induction of Glucose-6-Phosphate Dehydrogenase in Rat Hepatocyte Monolayers

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Rat hepatocytes in monolayer culture were utilized to determine if the decrease in glucose-6-phosphate dehydrogenase (G6PD) activity resulting from the ingestion of fat can be mimicked by the addition of fatty acids to a chemically, hormonally defined medium. G6PD activity in cultured hepatocytes was induced several-fold by insulin. Dexamethasone or T3 did not amplify the insulin induction of G6PD. Glucose alone increased G6PD activity in cultured hepatocytes from fasted donors by nearly 500%. Insulin in combination with glucose induced G6PD an additional two-fold. The increase in G6PD activity caused by glucose was greater in hepatocytes isolated from 72 hr-fasted rats as compared to fed donor rats. Such a response was reminiscent of the "overshoot" phenomenon in which G6PD activity is induced well above the normal level by fasting-refeeding rats a high glucose diet. Addition of linoleate to the medium resulted in a significant suppression of insulin's ability to induce G6PD, but linoleate had no effect on the induction of G6PD activity by glucose alone. A shift to the right in the insulin-response curve for the induction of G6PD also was detected for the induction of malic enzyme and acetyl-CoA carboxylase. Arachidonate (0.25 mM) was a significantly more effective inhibitor of the insulin action than linoleate was. Apparently rat hepatocytes in monolayer culture can be utilized as a model to investigate the molecular mechanism by which fatty acids inhibit the production of lipogenic enzymes. In part, this mechanism of fatty acid inhibition involves desensitization of hepatocytes to the lipogenic action of insulin.
***Lipids 23,* 36–41 (1988).**

Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.4.9), the rate-determining enzyme of the hexose monophosphate shunt, is a source of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for cellular lipogenesis. The activity and synthesis of hepatic G6PD in vivo is affected by changes in insulin, glucocorticoid and thyroid hormone availability (1–7). Diabetes mellitus results in a pronounced loss of hepatic G6PD activity, which is restored by insulin administration (1). Adrenalectomy prevents the overshoot response of G6PD in fasted-refed rats (5), and thyroidectomy attenuates the stimulation of G6PD by dietary carbohydrate (6,7). The role of specific hormones in the modulation of G6PD synthesis has been identified utilizing cultured hepatocytes in a defined hormonal environment. Insulin and glucocorticoids are key regulatory hormones that enhance both G6PD synthesis and mRNA concentration (8–12). Thyroid hormone, in contrast to its purported stimulatory action in vivo (3,6,7), has no affect on the level of G6PD in isolated hepatocytes (8,9).

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Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

Dietary fat is one of many factors that alter the hepatic content of G6PD. The induction of G6PD by dietary carbohydrate is impaired by the addition of fat, specifically polyenoic fatty acids to the diet (2,4,13). Similarly the fast-refeed overshoot of G6PD is attenuated by dietary corn oil (14). The inhibitory effect of dietary fats may be a direct regulation of hepatic gene expression. Alternatively, dietary fats may alter the concentration or modulate the action of key lipogenic hormones. Primary hepatocyte cultures provide a model to study the interaction of fatty acids with hormones on the induction of G6PD. In this communication, we report that the unique stimulatory action of insulin on G6PD is attenuated by the addition of fatty acids to the culture medium.

MATERIALS AND METHODS

Materials. Collagenase (EC 3.4.99.5) type IV was obtained from Sigma Chemical Co. (St. Louis, MO). Insulin was provided generously by Walter N. Shaw, Lilly Research Laboratories (Indianapolis, IN). Waymouth MB 752/1 medium containing glutamine and no bicarbonate and the newborn calf serum were purchased from Grand Island Biological Co. (Grand Island, NY). Tissue culture plates were obtained from Falcon Plastics (Oxnard, CA). Fatty acids were obtained from NuChek Prep (Elysian, MN). Dexamethasone-hexadrol phosphate was obtained from Organon (West Orange, NJ). Triiodothyronine (T3) and all other chemicals were purchased from Sigma Chemical Co. Collagen for coating tissue culture plates was isolated from rat tails as recommended by Michalopolous and Pitot (15). Sprague-Dawley rats were purchased from Harlan Laboratories (Madison, WI).

Liver cell isolation and culture. Liver cells were isolated and cultured as previously described (16). The hepatocyte donors were rats that had been fasted for 72 hr or rats fed laboratory chow. Fasted donors were compared to fed donors in order to ascertain if fatty acid suppression of the G6PD induction that occurs with refeeding in vivo was reproducible in vitro, if G6PD induction was less variable and more pronounced in fasted rats, and if a shorter prehormone culture period could be employed in order to shorten the time of cell culture. The isolated hepatocytes were washed into cold, well-oxygenated culture Hi/Wo/Ba medium (16) and plated onto dry collagen-coated tissue culture plates at a density of 3×10^6 cells per 60 mm plate and in a total volume of 3 ml of medium. The cells were allowed to attach to the plates for four hr in the presence of 4% newborn calf serum (v/v). After the attachment period, cells were maintained for an additional 24 hr (fasted donors) or 48 hr (fed donors) in a serum- and hormone-free medium prior to hormonal additions. The hepatocytes were maintained in culture for 96–120 hr with media changes occurring at 24 hr-intervals. The concentrations of hormones and fatty acids are indicated in the Results section. The Hi/Wo/Ba medium utilized in our studies consists of Waymouth MB 752/1 containing 28 mM glucose, glutamine (6.8 mM),

alanine (0.4 mM), serine (0.5 mM), $NaHCO_3$ (26 mM), bovine serum albumin (0.2%). Thus, a glucose stimulus for enzymatic induction was present during the 24 or 48 hr hormone-free period, except in the experiments of Figure 2 in which the inducing action of glucose alone was investigated. In these studies, glucose was absent during the 24 hr hormone-free period.

The viability of freshly isolated liver cells and cultured hepatocytes was ascertained by ATP measurements (17) and trypan-blue exclusion. Freshly isolated liver cells were 95% viable as determined by trypan-blue exclusion. ATP concentration was routinely between 0.8 and 1.2 μmol/mg DNA in the freshly isolated and cultured cells.

The cultured cells were harvested by scraping the hepatocytes from the plates with 1 ml of cold-buffered digitonin solution: Mops, pH 7.0 (20 mM), dithiothreitol (1 mM), ethylenediamine tetraacetic acid (EDTA) (2.5 mM), mannitol (250 mM) and digitonin (5 mg/ml). After a five min incubation on ice, the cell digitonin mixture was microfuged for five min. The supernate was utilized for G6PD measurements, and the cell pellets analyzed for DNA (18).

Glucose-6-phosphate dehydrogenase activity. Enzyme activity was determined in cell supernatants. NADP reduction was followed fluorimetrically in an assay system that consisted of Tris-acetate, pH 7.0 (50 mM), dithiothreitol (0.5 mM), NADP (0.5 mM) and glucose-6-phosphate (2 mM). One unit of enzyme activity is defined as the formation of 1 μmol of NADPH per min at 30 C. This assay assumes that under our conditions, 6-phosphogluconate dehydrogenase activity was not rate-limiting in the reaction. In support of such an assumption, Kelley et al. (19) reported that hepatocytes prepared from rat donors in a metabolic state comparable to that of the rats in our studies possessed 6-phosphogluconate dehydrogenase activity that significantly exceeded the activity of G6PD. Therefore, it is unlikely that 6-phosphogluconate dehydrogenase activity affected our G6PD activity determinations. Furthermore, this means that 2 μmol of NADPH are assumed to be produced per μmol of glucose-6-phosphate utilized.

Other assays. Free fatty acids were bound to bovine serum albumin as previously described (16). The albumin had been washed previously with charcoal and florisil to remove residual lipid. Cultured cells not receiving fatty acids received an equivalent amount of albumin.

RESULTS

Induction of G6PD in hepatocyte monolayers prepared from fed and fasted donor rats. Insulin in the presence of 28 mM glucose markedly enhanced G6PD activity in cultured rat hepatocytes prepared from fed or fasted donors (Fig. 1). Dexamethasone did not amplify the insulin induction of G6PD (Fig. 1). Furthermore, T_3 alone or in combination with insulin or insulin plus dexamethasone did not increase G6PD activity regardless of the nutritional status of the hepatocyte donor (data not shown).

Much of the apparent insulin response in liver cells was due to glucose per se (Fig. 1). As an example, hepatocytes from fasted donor rats cultured for 72 hr with 28 mM glucose and without hormones possessed G6PD activity

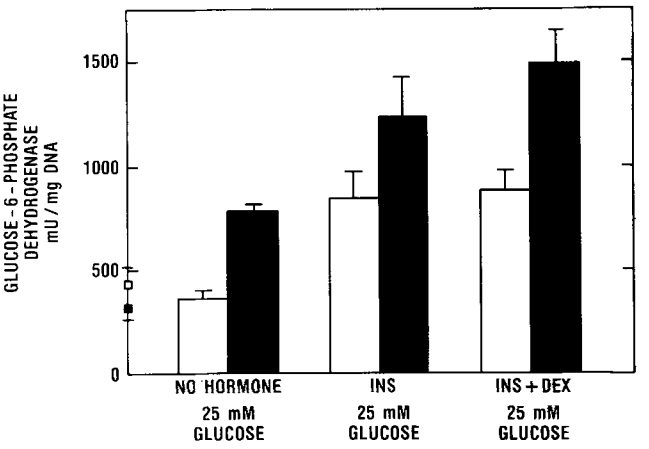

FIG. 1. Induction of G6PD activity in rat hepatocyte monolayers from fed and fasted donors. Hepatocyte monolayers were prepared from fed or 72 hr-fasted rats and maintained in culture as described in the Methods section. The medium contained 28 mM glucose during the 24 hr and 48 hr prehormone treatment culture period for the cells from fasted and fed donors, respectively. The initial G6PD activity at the time of cell isolation is depicted by the open (fed donors) and solid (fasted donors) squares on the vertical axis. The concentration of insulin and dexamethasone was 10^{-6} M. The period of hormone treatment was 72 hr. Open and solid bars represent hepatocytes from fed and fasted donor rats, respectively. Each value represents the mean of three to seven separate culture preparations with three to four plates/culture. Statistical analysis using two-way analysis of variance and F-test revealed significant ($P < 0.05$) effects of hormone and fasting but no hormone × fasting interaction (38).

that was 190% greater than the activity in freshly isolated cells (Fig. 1).

An increase of G6PD activity during culture did not occur when hepatocyte monolayers were maintained in glucose-free medium, and in fact G6PD activity declined from 191 mU/mg DNA in freshly isolated cells to 48 mU/mg DNA after 96 hr in culture (Fig. 2). Glucose induction of G6PD was nearly maximal with a glucose concentration of 5 mM, which approximately is equivalent to the level of circulating blood glucose. The glucose increment accounted for 40 to 50% of the apparent insulin-stimulated rise of G6PD activity in the hepatocytes from fasted donors. In contrast, G6PD activity was not induced by glucose in hepatocytes prepared from fed donors (Fig. 1, compare the vertical axis value with the column for no hormone-fed rat). Consequently, when the insulin induction was corrected for G6PD activity in the absence of hormones, the insulin increment was the same in hepatocytes from both fed and fasted donors, 484 mU/mg DNA vs 456 mU/mg DNA, respectively.

The glucose induction of G6PD apparently was greater in hepatocytes from fasted as compared to fed donors (Fig. 1). However, this conclusion is equivocal because the 24 hr longer prehormone culture period for cells from the fed donor may have been a factor in the apparent responsive difference between fed and fasted donors.

Fatty acid inhibition of the hormonal induction of G6PD. Dietary fatty acids are effective inhibitors of G6PD activity in vivo (2,4,13,14). Therefore, we hypothesized that the induction of G6PD in hepatocyte cultures would be attenuated by the addition of free fatty acids to the culture medium.

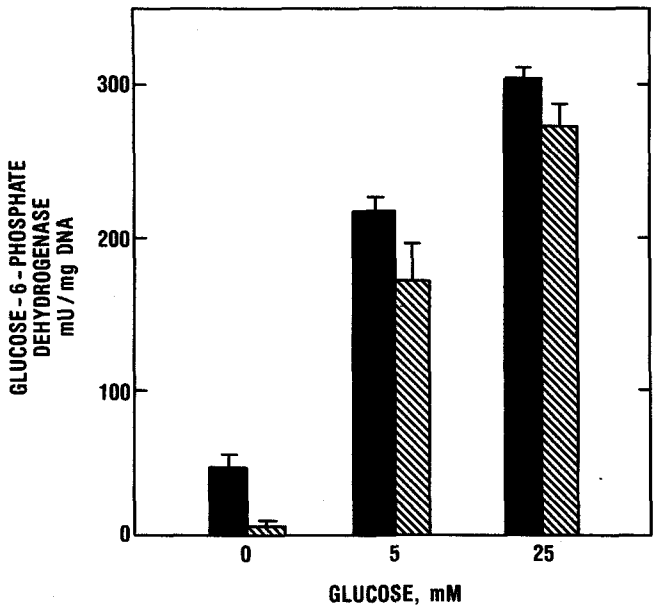

FIG. 2. Induction of G6PD by glucose in hepatocyte monolayers prepared from 72 hr-fasted rats. Hepatocyte monolayers were prepared from 72 hr-fasted rats and maintained in culture as described in the Methods section. The medium contained no glucose during the 24 hr prelinoleate treatment culture period. The concentration of glucose during the linoleate treatment phase is depicted in the figure. Hepatocytes were maintained in a media containing no fatty acid (solid bars) or 0.25 mM albumin-bound linoleate (hatched bars). Hepatocytes were harvested 72 hr after glucose addition. G6PD activity in freshly isolated hepatocytes was 191 mU/mg DNA. Each bar represents the mean of three determinations. Two-way analysis of variance and F-test (38) revealed a significant ($P < 0.05$) induction by glucose but no effect of fat.

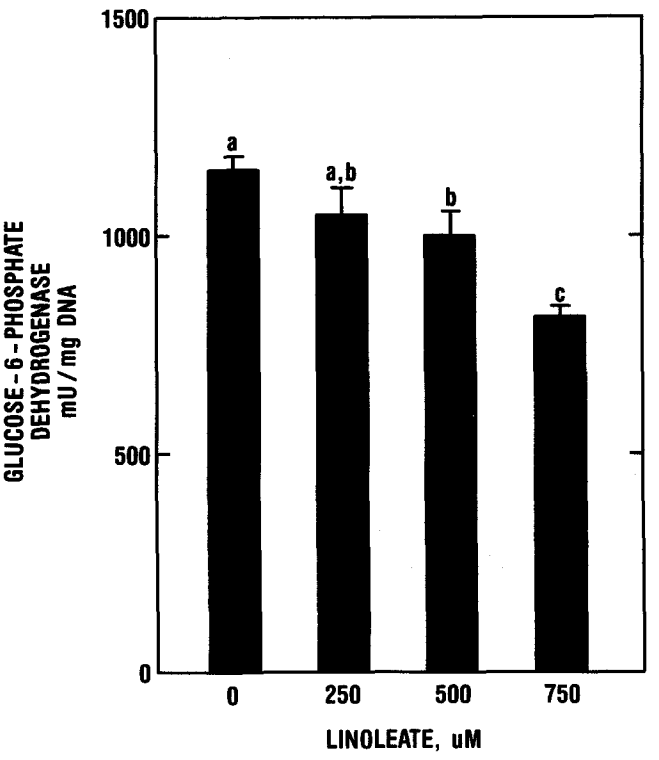

FIG. 3. Influence of linoleate on the induction of G6PD activity by insulin plus dexamethasone. Hepatocyte monolayers were prepared from 72 hr-fasted rats and maintained in culture as described in the Methods section after the hepatocytes had been maintained in culture for 24 hr in a medium containing 28 mM glucose and free of serum or hormones. After this 24 hr period, 10^{-6} M insulin/dexamethasone and the indicated concentrations of albumin-bound linoleate were added to the medium. Hepatocytes were maintained an additional 72 hr. Each bar represents the mean of three determinations from a representative cell preparation. Values with different letters are significantly different ($P < 0.05$) as evaluated by one-way analysis of variance and Tukey's t-test (38).

Hepatocytes from fasted donors were utilized in order to investigate the effect of fatty acids on G6PD response to both glucose and hormones. Linoleate was chosen as the fatty acid for primary investigation because it is not cytotoxic (16), maintains the linoleate content of the hepatocyte membrane (unpublished data) and more effectively suppresses the synthesis of lipogenic enzymes in the intact rat (2,13). The initial studies suggested fatty acids did not inhibit G6PD induction, i.e. glucose induction of G6PD was not significantly suppressed by the inclusion of linoleate (250 μM) in the culture medium (Fig. 2). Further, the induction of G6PD by 10^{-6} M insulin plus dexamethasone was not significantly decreased by 250 μM linoleate (Fig. 3). Palmitate, oleate, arachidonate, eicosapentaenoate or docosahexaenoate (250 μM) similarly were ineffective (1587, 1317, 1244, 1502 and 1361 mU/mg DNA, respectively vs 1159 mU/mg DNA without fatty acid). The induction of G6PD activity by insulin/dexamethasone was attenuated by levels of fatty acid in excess of 250 μM, but the extent of suppression was only 13% and 30% for 500 and 750 μM linoleate, respectively (Fig. 3). This inhibition was not a toxic effect of fatty acid on hepatocyte viability. Cellular ATP concentration was not lowered significantly by linoleate (Table 1).

It is possible that with 10^{-6} M insulin plus 10^{-6} M dexamethasone, the stimulatory action of the hormones overwhelmed the inhibitory mechanism of the free fatty acids. Therefore, the influence of free fatty acid on the insulin response curve for the induction of G6PD activity was examined in the absence and presence of dexamethasone (Figs. 4, 5 and 6). In the presence of 10^{-6} M dexamethasone, free fatty acid had no effect on the insulin-dependent induction of hepatocyte G6PD activity (Fig. 4). However,

TABLE 1

Influence of Linoleate on Hepatocyte ATP Concentration

Linoleate, μM	ATP, nmol/mg DNA
0	1.26 ± 0.11
250	0.96 ± 0.01
500	1.11 ± 0.04
750	0.86 ± 0.03

After the hepatocytes (isolated from fasted donors) were maintained in culture for 24 hr in the absence of hormone or serum (Hi/Wo/Ba, 28 mM glucose), the medium was changed to one containing 1 μM insulin and albumin-bound linoleate at the concentrations indicated. Each value is the average of three observations ± S.E. Fatty acid did not affect significantly the cellular ATP concentration as determined by one-way analysis of variance and F-test (38).

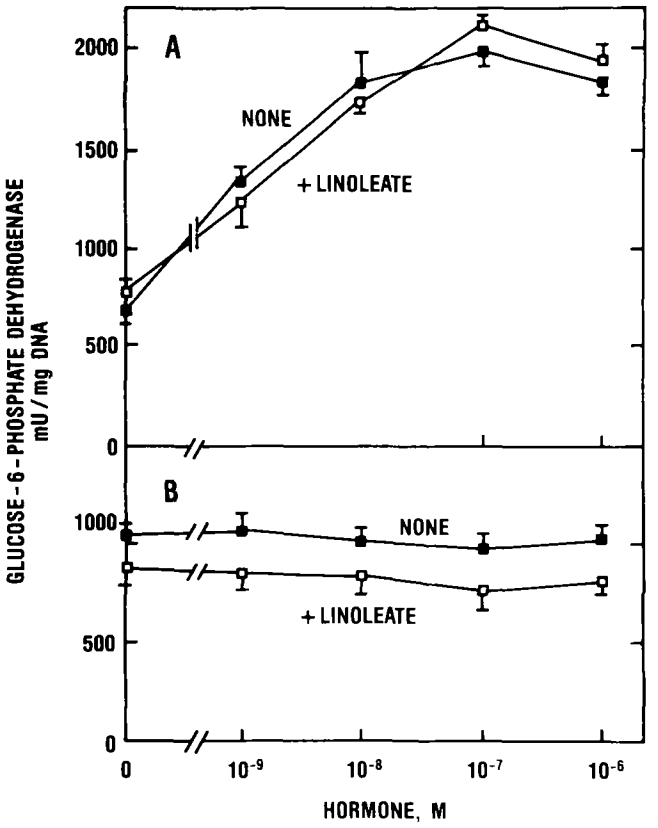

FIG. 4. Failure of linoleate to suppress G6PD induction in hepatocyte monolayers when the media contains 10^{-6} M dexamethasone (A) or insulin (B). Hepatocyte monolayers were prepared from 72 hr-fasted rats and maintained in culture as described in the Methods section. After the hepatocytes had been maintained in culture for 24 hr in a medium containing 28 mM glucose and no serum or hormones, insulin and dexamethasone were added to the medium. Figure 4A (Experiment 1) represents the response to varying insulin concentrations at a constant concentration of 10^{-6} M dexamethasone, and Figure 4B (Experiment 2) represents the response of G6PD to varying dexamethasone concentrations in the presence of 10^{-6} M insulin. Hepatocytes were harvested 72 hr after the addition of the hormone/linoleate supplements. Open squares and closed squares represent the induction of G6PD activity in the presence and absence of 0.25 mM albumin-bound linoleate. The initial activity of G6PD, i.e. 24 hr without serum or hormones, was 384 mU/mg DNA. Two-way analysis of variance indicated a significant effect of insulin ($P < 0.05$).

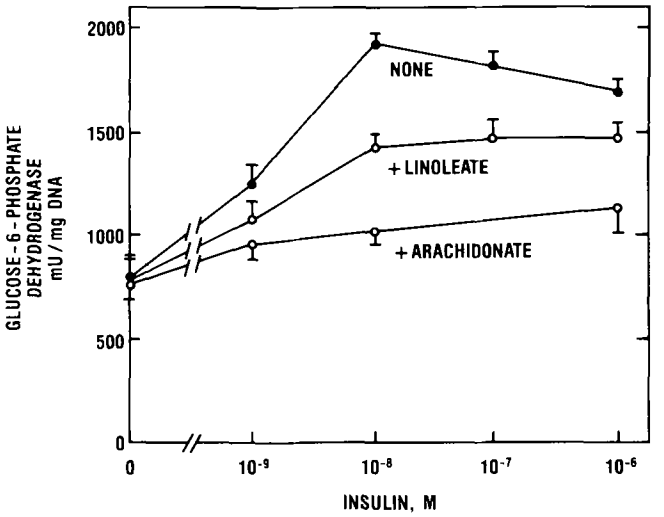

FIG. 5. Fatty acids impaired the insulin induction of hepatocyte G6PD when dexamethasone was deleted from the medium. Hepatocyte monolayers were prepared from 72 hr-fasted rats and maintained in culture as described in the Methods section. After the hepatocytes had been maintained in culture for 24 hr in a medium containing 28 mM glucose and no serum or hormone, insulin and fatty acids were supplemented to the media. Hepatocytes were harvested after being maintained an additional 72 hr. The concentration of albumin-bound linoleate and arachidonate was 0.25 mM. Each value represents the mean of three determinations. The induction of G6PD by insulin and the inhibition by fatty acid were significant ($P < 0.05$) as evaluated by a two-way analysis of variance and F-test. Arachidonate was significantly more inhibitory than linoleate ($P < 0.05$).

when dexamethasone was omitted from the media 250 μM albumin-bound linoleate significantly ($P < 0.05$) decreased the insulin-dependent induction of G6PD activity (Fig. 5). The shift of the insulin response curve to the right, which resulted from the supplementation of linoleate to the media, implies that linoleate decreased insulin sensitivity in cultured hepatocytes. A significant shift to the right in the response curve for insulin also was observed for the activities of acetyl-CoA carboxylase and malic enzyme (Figs. 6A and 6B). Arachidonate, the elongation and desaturation product of linoleate, was a significantly more effective inhibitor of G6PD induction than was linoleate (Fig. 5).

DISCUSSION

The hyperlipogenic condition induced by insulin in hepatocyte monolayers is accompanied by an increase in G6PD activity and in acetyl-CoA carboxylase (16) and malic enzyme activities (20,21). G6PD is uniquely sensitive to insulin in a manner generally unaffected by glucocorticoids (Figs. 1 and 4). The enhancement of G6PD activity by insulin is potentially via an increase in both pre- and post-translational events (22). In this regard, insulin enhancement of G6PD synthetic rate in primary rat hepatocyte monolayers was accompanied by an increase in G6PD-mRNA concentration (10,11).

While insulin is essential to maximizing the induction of lipogenic enzymes in cultured hepatocytes, glucose also appears to play a key role in this process, particularly in hepatocytes from fasted donors (Figs. 1 and 2). The fact that the glucose induction of G6PD activity was near maximum at the physiological concentration of glucose, i.e. 5 mM, indicates that glucose may play a significant role as a determinant of G6PD activity in vivo. In addition, the inductive effect of glucose in hepatocytes from fasted rats suggests that glucose per se is an important factor in the enzyme "overshoot" phenomenon of G6PD associated with fasting and refeeding carbohydrate (5,13). This effect of glucose may be at the level of transcription (23) and translation (24). Glucose treatment of hepatocytes was reported to increase the expression of several mRNAs (23) while accelerating the synthesis of G6PD (24). The stimulus for glucose induction of G6PD may be related to the undefined carbohydrate signal proposed by Mariash and Oppenheimer (20). Alternatively, the glucose mechanism may be nonspecific in the sense that it is serving as a source of energy or glycosylation substrate rather than being a specific regulator of gene expression. Nevertheless, a role for glucose as a hormone-independent

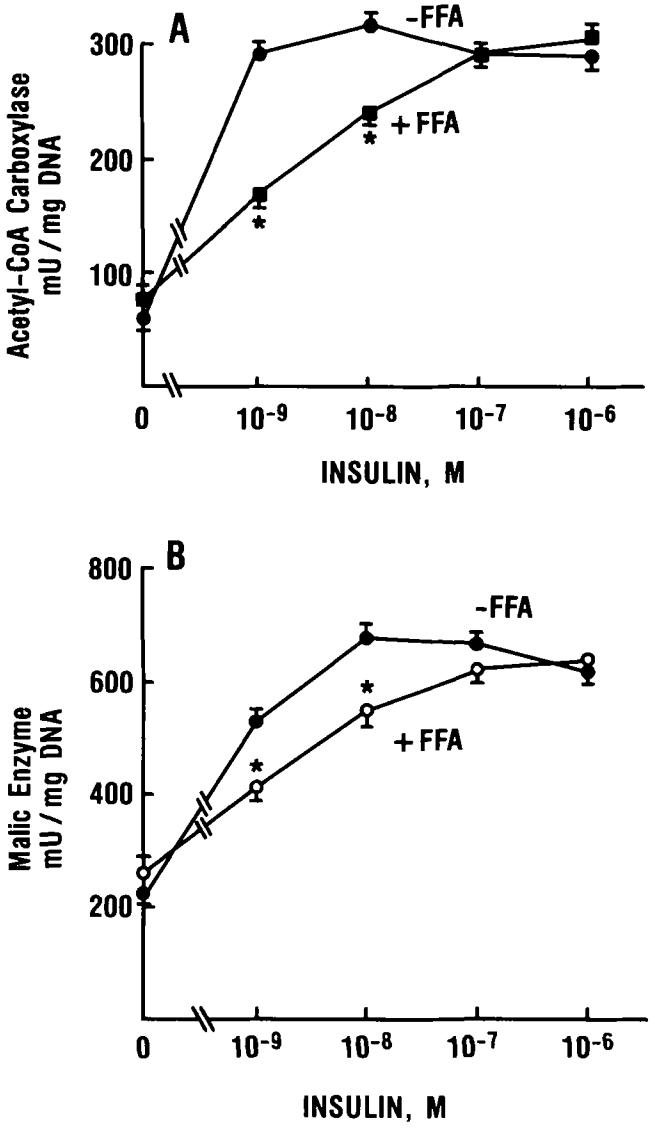

FIG. 6. Linoleate impairment of the insulin induction of hepatocyte acetyl-CoA carboxylase (A) and malic enzyme (B). Hepatocyte monolayers were prepared and maintained as described in the legend for Figure 5 except that the media of hepatocytes used to determine the activity of acetyl-CoA carboxylase contained 10^{-6} M dexamethasone. The concentration of linoleate was 0.25 mM. Each value represents the mean of three determinations. The asterisk indicates values are significantly ($P < 0.05$) lower than the activity in the absence of fatty acid.

inducer of G6PD may be teleologically advantageous because of the need for pentoses in cellular RNA/DNA synthesis.

In addition to a need for carbohydrate, adrenalcorticoids reportedly are necessary for the hyperinduction of G6PD activity in the intact animal (5). However, our studies with hepatocyte monolayers indicate that the G6PD induction primarily is a response to glucose and insulin (Figs. 1 and 2). Inclusion of dexamethasone in the media did not further increase the induction of G6PD. In contrast, dexamethasone has been reported to amplify the insulin induction of G6PD by increasing hepatocyte content of G6PD-mRNA without enhancing G6PD-mRNA translation (10). Our inability to detect an amplification of the insulin action may be the consequence of differences in culturing techniques. Specifically, our method for culturing hepatocytes utilizes a four-hr attachment period in a media containing 4% newborn calf serum.

Possibly the serum contains sufficient glucocorticoids or other stimulatory factors to eliminate the dexamethasone amplication of the insulin effect on G6PD expression. However, neither our hepatocyte model nor that of Stumpo and Kletzien (10) has defined completely the optimum conditions for G6PD expression in hepatocyte monolayers because maximal G6PD activity in cultured hepatocytes (Figs. 1 and 4; [8–10]) does not reach the level achieved in vivo (5). Apparently, additional stimuli (25), e.g. epidermal growth factor (25), are necessary for optimum hepatic G6PD induction.

Although rat hepatocytes in monolayer culture have limitations, they have provided a model in which to pursue the mechanism by which dietary fats inhibit the induction of lipogenic enzymes. One of the apparent mechanisms involves a fatty acid suppression of insulin action (Figs. 5 and 6). Free fatty acid, i.e. linoleate, reduced the sensitivity of hepatocytes to insulin as judged by the expression of G6PD, acetyl-CoA carboxylase, and malic enzyme activities. This effect occurred at physiological fatty acid concentrations and was not due to hepatocellular toxicity of the linoleate (Table 1). The linoleate/insulin interaction in the hepatocyte monolayers appears to be in disagreement with our recent report (26) that suppression of lipogenesis by dietary fat is independent of insulin. The explanation for this difference may reside in the fact that while linoleate continued to be a more effective inhibitor of lipogenesis in severely diabetic rats fed fructose, complete B-cell ablation may not have been achieved in our in vivo study (26). Mariash and Oppenheimer (20) have stated that the metabolism of fructose and its induction of lipogenic enzymes is not insulin-independent and does not occur in rats in whom serum insulin levels are undetectable. Hepatocytes in primary culture may provide a superior model for defining nutrient/hormone interactions because of the difficulties encountered with in vivo hormone ablation-replacement approaches.

Arachidonic acid was significantly more effective than linoleate (Fig. 5), which suggests the inhibitory action of linoleate may be a consequence of its conversion to arachidonate. In this regard, an inhibitor of linoleate elongation and desaturation prevented the dietary linoleate inhibition of lipogenic enzymes (27,28). However, supplementation of arachidonate to the diet reinstated the suppression of lipogenic enzymes (28). Although prostaglandins have been proposed as mediators of the linoleate/arachidonate inhibition, their role is equivocal because polyenoic fatty acids that are not prostanoid precursors, e.g. ω-3 fatty acids, also reduce the hepatic content of lipogenic enzymes (4).

It is possible that the fatty acid inhibition of the insulin induction of G6PD may have been the consequence of direct inhibition of transcription or translation or an indirect mechanism involving interference with the transduction of the insulin signal. The observation that fatty acids only interfered with insulin function as evaluated by the activities of G6PD, acetyl-CoA carboxylase and malic enzyme suggests that the mechanism of fatty acid inhibition may be indirect, i.e. reduced insulin binding and/or impaired transduction of insulin events

through the plasma membrane. In this respect, liver plasma membranes isolated from rats fed a high-fat diet bind less insulin bind insulin and generate less of the putative insulin second message (29,30). Such insulin insensitivity is attributed to altered membrane glycoprotein caused by reduced activity of several hepatic glycosyltransferases (31,32). Long-term culturing of hepatocytes with linoleate or arachidonate may be speculated to elicit a similar reduction in glycoprotein metabolism.

If fatty acids impaired the production of the insulin second message, a consequence could be reduced pyruvate dehydrogenase activation and subsequently less synthesis of the purported glucose signal that stimulates synthesis of lipogenic enzymes (30,33). Alternatively, impaired insulin binding and/or decreased transduction of the insulin signal hepatocytes cultured with fatty acids could lead to reduced insulin-receptor tyrosine kinase activity and decreased insulin mediated phosphorylation of S6-ribosomal protein (34), i.e. decreased translation rate. Such mechanisms are purely speculative but are consistent with the reported specific inhibition of fatty acid synthetase synthesis (35) and malic enzyme-mRNA translation by dietary linoleate (36). Furthermore, these hypotheses provide testable theories for the investigation of the mechanism by which dietary polyunsaturated fats suppress the synthesis of lipogenic enzymes (2).

Finally, an explanation for the alleviation of the fatty acid inhibition of G6PD-induction by dexamethasone may be that the effective intracellular free fatty acid concentration is decreased by dexamethasone as a result of phosphatidate phosphohydrolase induction (37). The consequence of this would be diversion of fatty acids into triglyceride and phospholipid (37) and a decrease in pool size of the putative fatty acid inhibitor. This effect of dexamethasone likely explains why in our previous studies, a suppression of acetyl-CoA carboxylase by free fatty acids was not discernible (16). Alternatively, dexamethasone simply may have increased the amount of G6PD-mRNA as a result of enhanced G6PD-mRNA transcription (10,11). Elucidation of the mechanism by which dexamethasone interferes with the fatty acid inhibition of lipogenic enzymes will require quantifying the level of mRNAs and rates of transcription. Certainly, our ability to develop a hepatocyte model that responds to the intact rat will greatly facilitate elucidation of the mechanism by which fatty acids suppress the production of lipogenic enzymes.

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REFERENCES

1. Gibson, D.M., Lyons, R.T., Scott, D.F., and Muto, Y. (1972) *Adv. Enzyme Reg. 10,* 87–204.
2. Toussant, M.J., Wilson, M.D., and Clarke, S.D. (1981) *J. Nutr. 111,* 146–153.
3. Miksicek, R.J., and Towle, H.C. (1982) *J. Biol. Chem. 257,* 11829–11835.
4. Schwartz, R.S., and Abraham, S. (1982) *Biochim. Biophys. Acta 711,* 316–326.
5. Williams, B.H., and Berdanier, C.D. (1982) *J. Nutr. 112,* 534–541.
6. Freedland, R.A., Murad, S., and Hurvitz, A.I. (1968) *Fed. Proc. 27,* 1217–1222.
7. Mariash, C.N., Kaiser, F.E., Schwartz, H.L., Towle, H.C., and Oppenheimer, J.H. (1980) *J. Clin. Invest. 65,* 1126–1134.
8. Kurtz, J.W., and Wells, W.W. (1981) *J. Biol. Chem. 256,* 10870–10875.
9. Nakamura, T., Yoshimoto, K., Aoyama, K., and Ichihara, A. (1982) *J. Biochem. Tokyo 91,* 681–693.
10. Stumpo, D.J., and Kletzien, R.F. (1984) *Eur. J. Biochem. 144,* 497–502.
11. Yoshimoto, K., Nakamura, J., Niimi, S., and Ichihara, A. (1983) *Biochim. Biophys. Acta 741,* 143–149.
12. Winberry, L., Nakayama, R., Wolfe, R., and Holten, D. (1980) *Biochem. Biophys. Res. Commun. 96,* 748–755.
13. Herzberg, G.R. (1983) *Adv. Nutr. Res. 5,* 221–253.
14. Baltzell, J.K., and Berdanier, C.D. (1985) *J. Nutr. 115,* 104–110.
15. Michalopolous, G., and Pitot, H.C. (1975) *Exp. Cell Res. 94,* 70–78.
16. Salati, L.M., and Clarke, S.D. (1986) *Arch. Biochem. Biophys. 246,* 82–89.
17. Lowry, O.H., and Passoneau, J.V. (1972) in *A Flexible System of Enzymatic Analysis,* pp. 151–156, Academic Press, New York, NY.
18. Labarca, C., and Paigen, K. (1980) *Anal. Biochem. 102,* 344–352.
19. Kelley, D.S., Nelson, G.J., and Hunt, J.E. (1986) *Biochem. J. 235,* 87–90.
20. Mariash, C.N., and Oppenheimer, J.H. (1983) *Metab. 33,* 545–552.
21. Salati, L.M., and Clarke, S.D. (1986) *Fed. Proc. 45,* 1026.
22. Jones, E.A., Fritz, R.S., Prostko, C.R., and Kletzien, R.F. (1986) *Fed. Proc. 45,* 1584.
23. Topliss, D.J., Mariash, C.N., Seelig, S., Carr, F.E., and Oppenheimer, J.H. (1983) *Endocrinology 112,* 1868–1870.
24. Stumpo, D.J., and Kletzien, R.F. (1985) *Biochem. J. 226,* 123–130.
25. Yoshimoto, K., Nakamura, T., and Ichihara, A. (1983) *J. Biol. Chem. 258,* 12355–12360.
26. Wilson, M.D., Hays, R.D., and Clarke, S.D. (1986) *J. Nutr. 116,* 1511–1518.
27. Clarke, B.A., and Clarke, S.D. (1982) *J. Nutr. 112,* 1212–1219.
28. Abraham, S., and McGrath, H. (1977) *Lipids 12,* 446–449,
29. Sun, J.V., Tepperman, H.M., and Tepperman, J. (1977) *J. Lipid Res. 18,* 533–539.
30. Begum, N., Tepperman, H.M., and Tepperman, J. (1983) *Endocrinology 112,* 50–59.
31. Tepperman, H.M., Dewitt, J., and Tepperman, J. (1983) *Diabetes 32,* 412–415.
32. Tepperman, H.M., Silver, R., Dewitt, J., and Tepperman, J. (1981) *J. Nutr. 111,* 1734–1741.
33. Jarret, L., and Seals, D.R. (1979) *Science 206,* 1407–1408.
34. Cobb, M.H., and Rosen, O.M. (1983) *J. Biol. Chem. 258,* 12472–12481.
35. Flick, P.K., Chen, J., and Vagelos, P.R. (1977) *J. Biol. Chem. 252,* 4242–4249.
36. Schwartz, R.S., and Abraham, S. (1983) *Arch. Biochem. Biophys. 221,* 206–215.
37. Mangiapane, E.H., and Brindley, D.N. (1986) *Biochem. J. 233,* 151–160.
38. Steel, R.G.D., and Torrie, J.H. (1980) in *Principles and Procedures of Statistics. A Biometrical Approach,* 2nd ed., pp. 137–233, McGraw Hill Book Co., New York, NY.

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Metabolism of Sitosteryl β-D-Glucoside and Its Nutritional Effects in Rats

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[4-^{14}C]Sitosteryl β-D-glucoside, intragastrically administered to rats, was not absorbed by the intestinal mucosa. At three hr after the application, radioactivity was concentrated almost exclusively in the digesta of stomach, small intestine as well as cecum and colon, whereas only low proportions of radioactively labeled compounds were found in the various tissues of the gastrointestinal tract. Minor proportions of labeled metabolites of [4-^{14}C]sitosteryl β-D-glucoside, such as sitosterol and sitosteryl esters, were formed in the small intestine in vivo and in slices of small intestine in vitro. In the tissues of cecum and colon as well as the digesta derived from them, high proportions of labeled coprositostanol, i.e. 24α-ethyl-5β-cholestan-3β-ol, that obviously had been formed by bacterial degradation of the substrate were detected.

The feeding of sitosteryl β-D-glucoside (0.5 g/kg body weight × day) over a period of four weeks did not alter significantly body weights or organ weights of rats. Analyses of steryl lipids of the various organs and tissues confirmed the findings obtained with the radioactive substrate: neither sitosteryl β-D-glucoside nor sitosterol or sitosteryl esters derived therefrom had been transported in appreciable amounts to organs and tissues outside the alimentary canal during the feeding period. Minor proportions of unmetabolized sitosteryl β-D-glucoside were detected in the tissues of stomach and intestine, whereas large proportions of the substrate were found in feces of rats that had received the sitosteryl β-D-glucoside-containing diet; coprositostanol was found in feces of these animals in high proportions as well. Thus, the use of sitosteryl β-D-glucoside as emulsifier or preservative in food and feed does not appear to involve any risk. ***Lipids 23,*** **42-47 (1988).**

β-Glycosides of sitosterol and of other phytosterols as well as acylated steryl β-glycosides are ubiquitous lipid constituents of higher plants (1). Steryl β-glycosides are also detected in algae (2), bacteria (3), yeasts (4) and, more recently, in the epidermis of chickens (5). Obviously, these substances are ingested daily with human food.

The steryl glycolipids show desirable amphiphilic and antioxidative properties (6,7) and, therefore, they appear to be potentially useful as emulsifiers and preservatives in a great variety of foodstuffs. Little is known, so far, on the intestinal absorption, metabolism and nutritional effects of steryl β-glycosides. This study records the metabolic fate and nutritional properties of sitosteryl β-D-glucoside, the most abundant steryl glycolipid in various vegetables.

Abbreviations: GLC, gas liquid chromatography; ip, intraperitoneally; TLC, thin layer chromatography. The systematic nomenclature of the sterols referred to by trivial names is, cholest-5-en-3β-ol (cholesterol); 5α-cholestan-3β-ol (5α-cholestanol); 5β-cholestan-3β-ol (5β-cholestanol, coprostanol); 24α-methylcholest-5-en-3β-ol (campesterol); 24α-methyl-5α-cholestan-3β-ol (5α-campestanol); 24α-methyl-5β-cholestan-3β-ol (5β-campestanol, coprocampestanol); 24α-methyl-cholesta-5,22-dien-3β-ol (brassicasterol); 24α-ethylcholest-5-en-3β-ol (sitosterol, β-sitosterol); 24α-ethyl-5α-cholestan-3β-ol (5α-sitostanol); 24α-ethyl-5β-cholestan-3β-cholestan-3β-ol (5β-sitostanol, coprositostanol); 24α-ethylcholesta-5,22-dien-3β-ol (stigmasterol).

MATERIALS AND METHODS

Chemicals. Sitosterol was obtained from E. Merck (Darmstadt, FRG) and pentaacetyl β-D-glucose from Sigma Chemie (Deisenhofen, FRG). Dihydrositosterols were prepared by catalytic hydrogenation of sitosterol with PtO_2 in ethyl acetate. The resulting mixture of 5α-dihydrositosterol (5α-sitostan-3β-ol) and small amounts of 5β-dihydrositosterol (5β-sitostan-3β-ol, coprositostanol) was separated twice by thin layer chromatography (TLC) on silica gel with hexane/ethyl acetate (85:15, v/v), and the components were isolated. Aliquots of sitosterol and 5α-sitostan-3β-ol were converted enzymatically to sitost-4-en-3-one and 5α-sitostan-3-one, respectively, using cholesterol oxidase (E.C. 1.1.3.6) from *Nocardia erythropolis* (Boehringer-Mannheim, Mannheim, FRG). [4-^{14}C]Sitosterol, 2.13 GBq/mmol, was purchased from Amersham Buchler (Braunschweig, FRG).

Preparation of substrates. Sitosteryl β-D-(2,3,4,6-tetraacetyl)glucoside was synthesized by the reaction of sitosterol with acetobromoglucose (8) in the presence of mercury(II)cyanide (9). The reaction product was purified by column chromatography on silica gel with diethyl ether. Traces of mercuric ions were removed by filtration of a methanolic solution of sitosteryl β-D-(2,3,4,6-tetraacetyl)glucoside through "Chelex 100" ion exchange resin (Sigma Chemie). Alkaline hydrolysis of the peracetylated reaction product yielded sitosteryl β-D-glucoside that was crystallized from methanol. The composition of steryl moieties in the substrate was determined by GLC after acid hydrolysis (16%, campesterol; 4%, stigmasterol; 65%, sitosterol; 5%, other sterols; and 10%, 5α-stanols).

[4-^{14}C]Sitosteryl β-D-glucoside, 1.11 GBq/mmol, for in vitro incubations as well as [4-^{14}C]sitosteryl β-D-glucoside, 37 MBq/mmol, for in vivo experiments were synthesized in a similar manner from [4-^{14}C]sitosterol and purified by repeated TLC on silica gel with dichloromethane/methanol (9:1, v/v). The radiochemical purity of the labeled sitosteryl β-D-glucoside was better than 98%.

Animals. Female WISW rats (Winkelmann Versuchstierzucht, Borchen, FRG) weighing 170-190 g were maintained at 22 C and 60% humidity and given Altromin standard diet (Altromin International, Lage, FRG) containing per 1 g 0.3 mg sterols (10% cholesterol, 19% campesterol, 7% stigmasterol, 5% other sterols, and 8% 5α-stanols) and 0.07 mg steryl β-D-glycosides (18% campesterol, 8% stigmasterol, 65% sitosterol, 5% other sterols and 4% 5α-stanols). Water was given ad libitum throughout the experiment.

In vivo administration of [4-^{14}C]sitosteryl β-D-glucoside. Radioactive sitosteryl β-D-glucoside (37 MBq/mmol; 148 kBq/animal) in 0.2 ml of a vegetable oil was administered

intragastrically to three animals that had been fasted for 12 hr and then fasted for another three hr. Distilled water, 5 ml/animal, was injected intraperitoneally (ip) one hr after administration of the radioactive substrate in order to get larger volumes of urine. The animals were maintained in metabolic cages throughout the experiment. The cages were flushed with synthetic air, and the carbon dioxide formed by oxidative degradation of feed was removed from the gas flux by absorption in triethanolamine. Aliquots of this solution were used for the determination of radioactivity by liquid scintillation counting.

In vitro incubations with [4-^{14}C]sitosteryl β-D-glucoside. Slices of small intestine (10) and liver snips (11), 0.7 g fresh weight each, were incubated for six hr at 30 C with 37 kBq (1.11 GBq/mmol) [4-^{14}C]sitosteryl β-D-glucoside in 4 ml TCM 199 medium (Seromed, München, FRG) that was supplemented with 1 g $NaHCO_3$/l and 10 mg amphotericin B/l and 10×10^6 I.U. streptomycin/l. The incubations were stopped by the addition of 15 ml dichloromethane/methanol (1:2, v/v) followed by homogenization using an Ultra-Turrax blender (IKA-Werke, Staufen, FRG). Lipids were extracted from the incubation mixtures and separated by TLC on silica gel as described below.

Feeding experiment. The rats were weighed and divided into two groups of eight animals, each. Group 1 was given 1 ml of a vegetable oil/animal × day for four wk using a pharynx tube, group 2 received 0.1 g synthetic sitosteryl β-D-glucoside/animal × day that was emulsified by sonication in 1 ml vegetable oil containing around 6 mg sterols (33% campesterol, 9% brassicasterol, 48% sitosterol, 8% other sterols, and 2% 5α-stanols). Every week, the total feed consumption and the weight gain of each group were determined. Feces of the two groups were collected over a period of 24 hr every week.

Extraction of lipids. The animals of the metabolic study were killed by cervical dislocation three hr after administration of the radioactive substrate. They were exsanguinated by section of the aorta. Organs and tissues (stomach, intestine, liver, kidneys, heart, lungs, spleen, brain, femoral muscle tissue and adipose tissue from the perirenal region) of three animals quickly were removed from the carcasses and weighed; digesta were removed from the stomach and various parts of the intestine. Each type of tissue and digesta was pooled and homogenized; the total lipids were extracted according to an established procedure (12). Radioactivity was determined in the various fractions by liquid scintillation counting in a Tri Carb C 2425 instrument (Packard Instruments, Downers Grove, IL).

At the end of the feeding period, the animals of the nutritional experiment were killed, and organs and tissues rapidly were dissected as described above. Subsequently, the total lipids were extracted according to an established procedure (12).

Analysis of radioactive lipids. The total lipids of each tissue as well as of digesta of the various parts of the gastrointestinal tract were fractionated on layers of silica gel using hexane/diethyl ether (4:1, v/v) followed by hexane/ethyl acetate (1:1, v/v) up to two-thirds of the plates from the origin, and the distribution of radioactivity in the various fractions was determined with a Berthold Automatic TLC Analyzer LB 2832 (BF-Vertriebsgesellschaft, Wildbad, FRG). The fractions of radioactively labeled lipids each were isolated and eluted from the adsorbent with water-saturated diethyl ether. The rather polar lipids were further fractionated on layers of silica gel using dichloromethane/methanol (9:1, v/v), and the distribution of radioactivity in the various classes of polar lipids was determined. The radioactive lipid fractions that were tentatively identified by cochromatography with standards were isolated as described above.

Aliquots of the fractions of ^{14}C-labeled steryl glycosides were acetylated and the resulting peracetylated derivatives were identified by cochromatography with synthetic sitosteryl β-D-tetraacetylglucoside on layers of silica gel using dichloromethane/methanol (98:2, v/v). Alternatively, these fractions were subjected to acid hydrolysis (5), yielding radioactive sterols that were identified by cochromatography with sterol standards on layers of silica gel using hexane/diethyl ether (3:2, v/v).

Radioactive sterol fractions were acetylated; the resulting steryl acetates were purified by TLC on silica gel with hexane/diethyl ether (4:1, v/v) and isolated as described above.

The fractions containing ^{14}C-labeled steryl esters of long-chain fatty acids were subjected to alkaline hydrolysis. The resulting radioactive sterols were purified by TLC and isolated. The various fractions of radioactive sterols and steryl acetates were analyzed by gas liquid chromatography (GLC) as described below using the Packard Gas Proportional Counter 894 for detection of radioactivity.

The fraction of radioactively labeled 5β-stanols (R_f 0.32) isolated from the digesta of cecum and colon (13) was separated twice by TLC on silica gel with hexane/ethyl acetate (85:15, v/v) from Δ^5-sterols plus 5α-stanols (R_f 0.25) and tentatively identified by cochromatography with coprostanol. The fraction of 5β-stanols containing ^{14}C-labeled coprositostanol was isolated from the adsorbent and acetylated. Identification of radioactive coprositostanyl acetate was carried out by $AgNO_3$-TLC twice using hexane/benzene (5:2, v/v) as solvent system (14) followed by radio-GLC using the conditions described below.

Aliquots of radioactive Δ^5-sterols plus 5α-stanols from the digesta of cecum and colon were converted to the corresponding Δ^4-en-3-oxo- (R_f 0.37) and 5α-dihydro-3-oxo compounds (R_f 0.53), respectively, by incubation with cholesterol oxidase and identified by cochromatography with standards on silica gel layers with hexane/ethyl acetate (7:3, v/v).

Analysis of lipid samples from the feeding experiment. The total lipids extracted from organs and tissues of rats from groups 1 and 2 of the feeding experiment were isolated as described above. Aliquots of total lipids of the various organs and tissues were separated by TLC on silica gel with hexane/diethyl ether (4:1, v/v) followed by dichloromethane/methanol (9:1, v/v). The fractions containing steryl β-glycosides, sterols and sterol esters that tentatively had been identified by cochromatography with standards were isolated and converted to the various derivatives as described for the radioactive steroid fractions.

The various fractions of Δ^5-sterols, 5β-stanols, steryl acetates and 5α-dihydro-3-oxo steroids were analyzed by GLC on a Perkin Elmer F 22 instrument (Perkin Elmer Bodenseewerk, Überlingen, FRG) equipped with flame ionization detectors. Peaks were assigned on the basis of

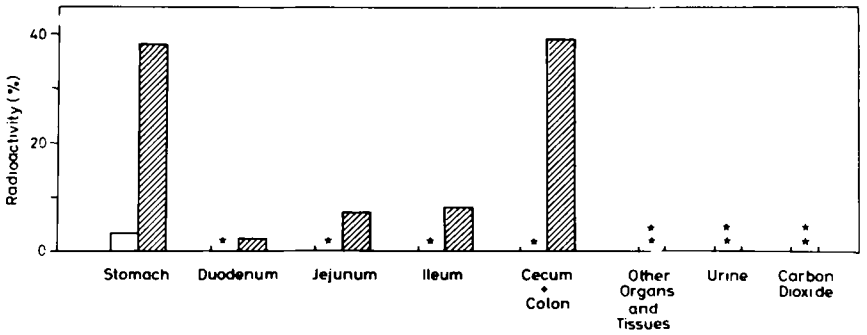

FIG. 1. Distribution of radioactivity from [4-^{14}C]sitosteryl β-D-glucoside in various organs and tissues as well as in excretion products of rats at three hr after intragastric application of the substrate. *, trace (<2%); ‡, not detected. For the stomach and the various parts of the intestinal tract, left: radioactivity in the tissues; right: radioactivity in the contents. Average from three animals.

retention times using reference compounds. Peak areas and retention times were determined using a Spectra Physics SP 4270 integrator (Spectra Physics, Darmstadt, FRG). The various sterol fractions were separated isothermically at 275 C (Δ^5-sterols, 5β-stanols) or 285 C (steryl acetates, 5α-dihydro-3-oxo steroids) on a glass column, 1.8 m × 3 mm, packed with OV-101 on Gas Chrom Q, 100–120 mesh (Applied Science Laboratories, State College, PA) with nitrogen as the carrier gas at a flow of 40 ml/min.

Statistical analysis. Statistical evaluation of differences between the values of body weights and organ weights of the control group and the group of animals that had received the diet containing sitosteryl β-D-glucoside was carried out using the Student's t-test for unpaired values.

RESULTS AND DISCUSSION

Intestinal absorption and metabolism of [4-^{14}C]sitosteryl β-D-glucoside. Initial studies were concerned with the intestinal uptake and metabolism of [4-^{124}C]sitosteryl β-D-glucoside that had been administered intragastrically to rats.

Figure 1 shows the distribution of radioactivity from [4-^{14}C]sitosteryl β-D-glucoside in various organs and tissues as well as in digesta of rats at three hr after application of this substrate. It is evident from these results that in all samples examined the radioactivity was detected predominantly in the contents of stomach and intestine. At three hr after stomach intubation, large proportions of radioactivity already had passed through the small intestine and were concentrated in the contents of cecum and colon. It is striking that only small proportions of radioactivity were associated with the intestinal tissues. Moreover, other organs and tissues such as liver, kidneys, heart, lungs, spleen, muscle, brain and adipose tissue as well as blood, obviously did not contain appreciable amounts of labeled substances.

The above findings indicate that minor proportions of the labeled substrate nonspecifically were adsorbed by the intestinal mucosa, whereas the major portion of the radioactive material passed without absorption through the alimentary canal. The virtual absence of radioactivity in organs and tissues other than gastrointestinal tissues may be attributed to a lack of transport of sitosteryl β-D-glucoside and its metabolites through the intestinal wall. In contrast, the structurally similar alkyl β-glycosides were found to be absorbed by the intestinal mucosa and, in part, transported as intact compounds to several other organs and tissues (15). Carbon dioxide formed by expiration as well as urine also are devoid of radioactivity.

The metabolism of sitosteryl β-D-glucoside was studied by examining the radioactive metabolites from extracts and digesta from various intestinal tissues of rats that had been given the radioactive substrate by stomach tube. These experiments were complemented by in vitro studies that involved incubations of [4-^{14}C]sitosteryl β-D-glucoside with slices of small intestine and liver snips. Analyses of radioactive reaction products revealed that large amounts of the labeled sitosteryl β-D-glucoside supplied as substrate remained unchanged both in the tissues of the gastro-intestinal tract, except cecum and colon, and in the in vitro incubations. Low proportions of radioactive metabolites of sitosteryl β-D-glucoside, mainly sitosterol and sitosteryl esters, were detected in the tissues of duodenum, jejunum, ileum and stomach (<0.5% of total radioactivity) as well as in the contents of the various parts of the small intestine (<1% of total radioactivity). These results are in good agreement with recent findings of investigators who tested the hemostatic effects of sitosteryl β-D-glucoside (16).

In addition, [4-^{14}C]sitosteryl β-D-glucoside was hydrolyzed by in vitro incubation with slices of small intestine at a rate of 0.8 nmol/g tissue × hr; small proportions of the resulting [4-^{14}C]sitosterol were acylated to the corresponding sitosteryl esters. However, only traces of radioactive sitosterol arose from the substrate in experiments with liver snips in vitro. It is evident from these data that the radioactive substrate is metabolized in both the in vivo and in vitro experiments at low rates.

From the data presented, it is obvious that the following reactions are involved in the metabolism of sitosteryl β-D-glucoside in the small intestine and liver of rats. Radioactive sitosterol is liberated by hydrolytic cleavage from [4-^{14}C]sitosteryl β-D-glucoside catalyzed by β-glucosidases that are present in the small intestine and liver (17–19). Substrate specificity of these enzymes for the steryl β-glycoside used seems to be rather low as compared to alkyl β-glycosides, e.g. (15). This is evident from the low proportions of radioactive sitosterol formed

during the experiments in vivo and in vitro. Moreover, the apparent absence of radioactive sitosterol in rat tissues other than those of the gastrointestinal tract is consistent with recent findings that the intestinal absorption of sitosterol and other plant sterols including 5α-stanols is limited compared to the fairly high absorbability of cholesterol (20–24).

In slices of the small intestine but not in liver snips, ^{14}C-labeled sitosterol is found to be further acylated, most probably by pancreatic cholesterol esterase and/or acyl-CoA:cholesterol acyltransferase (ACAT), yielding sitosteryl esters (22,25–27). The low rate of formation of sitosteryl esters from sitosterol (Table 1) is in good agreement with reports that esterification rate of cholesterol catalyzed by ACAT is by far higher than that of sitosterol (22).

Acyl transfer to position 6 of the sugar moiety of the substrate yielding sitosteryl (6-*O*-acyl)-β-D-glucoside obviously does not occur. Yet, 6-*O*-acylated steryl β-glycosides are common constituents of higher plants (1); they also are present in the epidermis of chickens (5). In addition, appreciable proportions of 1-*O*-alkyl-(6-*O*-acyl)-β-D-glucosides are formed in various tissues of mice from alkyl β-D-glucosides if these are used as subtrates (15).

However, in extracts from the tissues of cecum and colon as well as the corresponding digesta large proportions of radioactive metabolites of [4-^{14}C]sitosteryl β-D-glucoside that obviously had been formed by degradation of the substrate by microorganisms of the intestinal flora were detected. The main metabolites identified in the digesta of cecum and colon were coprositostanol, i.e. 24α-ethyl-5β-cholestan-3β-ol (8% of total radioactivity), and sitosterol (2% of total radioactivity). The radioactive tissue lipids of cecum and colon (<2% of total radioactivity) consisted about half of coprositostanol. This metabolite was identified by TLC and GLC as well as by its poor conversion to the corresponding 3-oxo steroids using cholesterol oxidase (28,29).

The formation of dihydrosterols is a well-known reaction occurring during the degradation of cholesterol and plant sterols such as sitosterol in mammals and microorganisms. Thus, 5α-stanols are synthesized in the liver of man and animals, whereas 5β-stanols such as coprostanol and coprositostanol are typical products of sterol metabolism in enteric microorganisms (13,30). The presence of small proportions of radioactive sitosterol and the obvious absence of radioactive sitost-4-en-3-one, which is a key intermediate in bacterial formation of 5β-stanols, reveals that this reaction is rate-limiting in the conversion of sitosterol to 5β-sitostanol. Similar results have been obtained for the transformation of sitosterol to 5α-sitostanol via sitost-4-en-3-one in rat liver (31).

The apparent absence of radioactive carbon dioxide in the expired air and of radioactive metabolites in the urine again show convincingly that [4-^{14}C]sitosteryl β-D-glucoside, given as substrate, virtually is not affected by oxidative degradation in the cells of rat organs and tissues.

Nutritional effects of sitosteryl β-D-glucoside. Steryl β-glycosides and acylated steryl β-glycosides occur in various vegetables such as potatoes and carrots at a level of about 2–8% of total lipids (32,33). Sitosteryl β-D-glucoside is found to be the most abundant steryl β-glycoside of higher plants. Absorption, transport and metabolism in animals (20–27,31,34–37) as well as pharmacological (31,38–40) and nutritional effects (23,37,41) of sitosterol are discussed in many publications, whereas little information is available on the corresponding properties of sitosteryl β-D-glycosides (16,42–44).

We have given 0.1 g sitosteryl β-D-glucoside/day to rats, i.e. 0.5 g/day × kg body weight, in order to study its nutritional effects. The intake of steryl β-D-glycosides from standard diet was around 1 mg/day × animal.

Figure 2 shows the effects of 0.1 g sitosteryl β-D-glucoside/day × animal on feed intake and weight gain of rats over a period of four wk. The average feed consumption of both groups of animals was 13–16 g per rat and day; the feed intake of the two groups of animals was quite similar. The results given in Figure 2 also show that the body weight of rats that had received sitosteryl β-D-glucoside statistically was not different ($P > 0.2$) from the body weight of animals in the control group.

The effects of sitosteryl β-D-glucoside on the weights of spleen, heart, kidneys and liver of rats are demonstrated in Figure 3. It is obvious that the weights of the various organs of the group of animals that had received the standard diet statistically was not different ($P > 0.2$) from the organ weights of animals that had been fed sitosteryl β-D-glucoside.

The proportions of sitosteryl β-D-glucoside and its metabolites, such as sitosterol and sitosteryl esters, in the lipids from the organs and tissues and from the feces of rats were determined by GLC. It was found that sterol fractions of various tissues of rats that had received the sitosteryl β-D-glucoside-containing diet and standard diet, respectively, have almost the same composition (approximately 95% cholesterol, 2–3% campesterol, 1–2% sitosterol and traces of other phytosterols). A similar

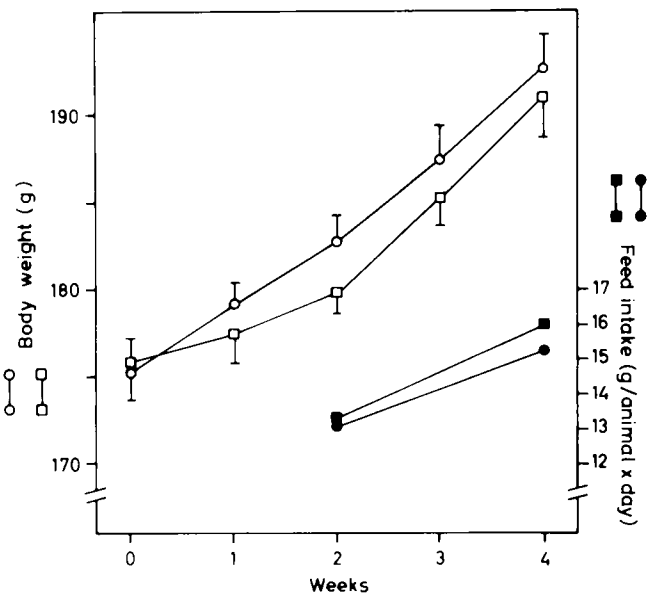

FIG. 2. Effects of standard diet or a diet containing sitosteryl β-D-glucoside administered for four wk on body weight and feed intake of rats. Values of body weight are mean ± SEM, n = 8; there are no statistically different values, $P > 0.2$; values of feed intake are mean. Standard diet, (○—○, ●—●); sitosteryl β-D-glucoside-containing diet, (□—□, ■—■).

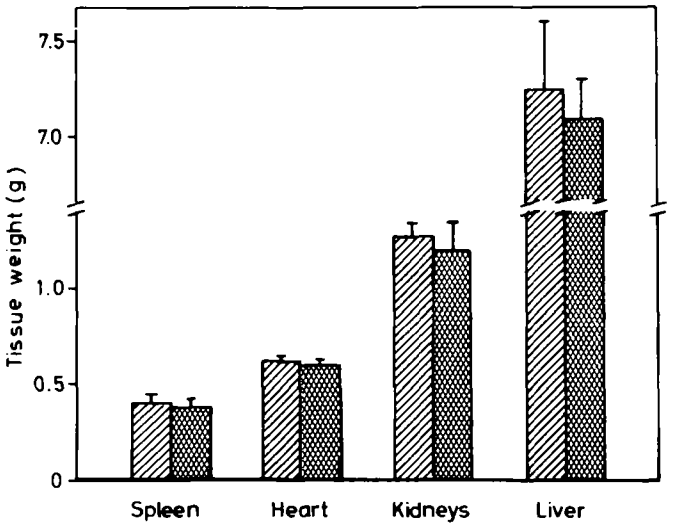

FIG. 3. Effects of standard diet or a diet containing sitosteryl β-D-glucoside administered for four wk on weights of various organs of rats. Values are mean ± SEM, n = 8; there are no statistically different values, P > 0.2. Standard diet, left column; sitosteryl β-D-glucoside-containing diet, right column.

composition of steryl moieties also was found in steryl esters isolated from various tissues of both groups of animals.

The content of Δ^5-sterols and 5α- and 5β-stanols of feces was quite similar in both groups of animals: feces of rats that had received the sitosteryl β-D-glucoside-containing diet were found to contain per 1 g: 1.1 mg Δ^5-sterols; 0.25 mg 5α-stanols; and 1.85 mg 5β-stanols, whereas 1.0 mg Δ^5-sterols, 0.25 mg 5α-stanols, and 1.75 mg 5β-stanols were detected in feces of the control group. Yet, the proportions of cholesterol and 5β-cholestanol decreased in fecal sterols of animals that had received the sitosteryl β-D-glucoside-containing diet and a concomitant increase of sitosterol and 5β-sitostanol was observed (Table 1). These findings are in good agreement with our observation that [4-^{14}C]sitosteryl β-D-glucoside was cleaved by highly active β-glycosidases of fecal microorganisms followed by hydrogen transfer to the resulting sitosterol, yielding 5β-sitostanol. The various 5α-stanols found in feces are derived predominantly from the feed (cf. sterol analyses given in the Materials and Methods section).

In the tissue lipids of the gastrointestinal tract of rats that had received the sitosteryl β-D-glucoside-containing diet, minor proportions (stomach 120 μg/g tissue; small intestine 1 μg/g tissue) of the unmetabolized substrate were found, whereas the lipids of other organs and tissues contain, if at all, only traces (<1 μg/g tissue) of steryl β-D-glycosides. In feces, major proportions of sitosteryl β-D-glucoside (around 1,300 μg/g feces dry weight) were detected at 20 hr after the last application of the substrate, whereas around 50 μg steryl β-D-glycosides derived from the standard diet were found per 1 g feces of the control group.

To summarize, it was found that [4-^{14}C]sitosteryl β-D-glucoside virtually is not absorbed in the gastrointestinal tract of rats. Yet, the substrate is cleaved at a low rate to ^{14}C-labeled sitosterol and glucose by the action of β-glycosidases of the small intestine. Remarkably, high proportions of radioactive coprositostanol derived from the substrate, which is caused most probably by bacterial transformation, are found in the tissues of cecum and colon and the corresponding digesta.

Sitosteryl β-D-glucoside, fed to rats at a level of 0.5 g/d × kg body weight for four wk, does not show results different from those of the control group with respect to feed intake, weight gain and organ weights. No ill effects are observed during this time and no abnormalities are detected in feces (color, blood, consistency) or urine (color, blood). Therefore, sitosteryl β-D-glucoside and other plant steryl β-glycosides may be of interest as potential emulsifiers and preservatives in food processing. An appreciable increase in fecal excretion of neutral steroids as found for various saponins (45,46) was not observed for sitosteryl β-D-glucoside.

TABLE 1

Composition of Sterols in Feces of Rats that had Received Sitosteryl β-D-Glucoside-containing Diet or Standard Diet

Sterols[a]		Composition (%) of various sterol fractions isolated from feces of rats that had received different diets	
		SG[b]	Standard
Cholesterol	Δ^5	6	9
Cholestanol	5α	<1	<1
	5β	11	19
Campesterol	Δ^5	7	7
Campestanol	5α	2	3
	5β	12	9
Sitosterol	Δ^5	19	15
Sitostanol	5α	5	4
	5β	32	27
Other sterols	Δ^5	3	3
	5α	<1	<1
	5β	3	3
Total sterols	Δ^5	35	34
	5α	8	8
	5β	57	58

[a] Δ^5, unsaturated sterols having a double bond at position 5; 5α, 5α-dihydrosterols (5α-stanols); 5β, 5β-dihydrosterols (5β-stanols, "coprostanols").

[b] SG, sitosteryl β-D-glucoside-containing diet.

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REFERENCES

1. Grunwald, C. (1975) *Annu. Rev. Plant Physiol. 26*, 209-236.
2. Duperon, R., Thiersault, M., and Duperon, P. (1983) *Phytochemistry 22*, 535-538.
3. Mayberry, W.R., and Smith, P.F. (1983) *Biochim. Biophys. Acta 752*, 434-443.
4. Kastelic-Suhadolc, T. (1980) *Biochim. Biophys. Acta 620*, 322-325.
5. Wertz, P.W., Stover, P.M., Abraham, W., and Downing, D.T. (1986) *J. Lipid Res. 27*, 427-435.

6. Liebenberg, R.W. (1972) Brit. Patent 1,298,047.
7. Ivanov, S.A., Seher, A., and Schiller, H. (1979) *Fette. Seifen. Anstrichm. 81,* 105-107.
8. Jeremias, C.G., Lucas, G.B., and MacKenzie, C.A. (1948) *J. Am. Chem. Soc. 70,* 2598.
9. Weber, N. (1977) *Chem. Phys. Lipids 18,* 145-148.
10. Elliot, K.A.C. (1955) in *Methods in Enzymology* (Colowick, S.P., and Kaplan, N.O., eds.) Vol. I, pp. 3-9, Academic Press, New York, NY.
11. Pollard, M.R., and Dutton, G.J. (1982) *Biochem. J. 202,* 469-473.
12. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
13. Rosenfeld, R.S., and Hellman, L. (1971) *J. Lipid Res. 12,* 192-197.
14. Heftmann, E. (1976) *Chromatography of Steroids,* Elsevier Scientific Publ., Amsterdam, New York, NY.
15. Weber, N., and Benning, H. (1984) *J. Nutr. 114,* 247-254.
16. Seki, J., Okita, A., Watanabe, M., Nakagawa, T., Honda, K., Tatewaki, N., and Sugyama, M. (1985) *J. Pharm. Sci. 74,* 1259-1264.
17. Robinson, D. (1956) *Biochem. J. 63,* 39-44.
18. Daniels, L.B., Coyle, P.J., Chiao, Y.-B., Glew, R., and Labow, R.S. (1981) *J. Biol. Chem. 256,* 13004-13013.
19. LaMarco, K.L., and Glew, R.H. (1986) *Biochem. J. 237,* 469-476.
20. Hassan, A.S., and Rampone, A.J. (1979) *J. Lipid Res. 20,* 646-653.
21. Grundy, S.M. (1983) *Annu. Rev. Nutr. 3,* 71-96.
22. Buchmann, M.S., and Clausen, O.P. (1986) *Lipids 21,* 738-743.
23. Tso, P. (1985) *Adv. Lipid Res. 21,* 143-186.
24. Tilvis, R.S., and Miettinen, T.A. (1986) *Am. J. Clin. Nutr. 43,* 92-97.
25. Watt, S.M., and Simmonds, W.J. (1981) *J. Lipid Res. 22,* 157-165.
26. Tavani, D.M., Nes, W.R., and Billheimer, J.T. (1982) *J. Lipid Res. 23,* 774-781.
27. Vahouny, G.V., Connor, W.E., Subramaniam, S., Lin, D.S., and Gallo, L.L. (1983) *Am. J. Clin. Nutr. 37,* 805-809.
28. Smith, A.G., and Brooks, C.J.W. (1976) *J. Steroid Biochem. 7,* 705-713.
29. Kirtchevsky, D., and Tepper, S.A. (1979) *Clin. Chem. 25,* 1464-1465.
30. Smith, L.L. (1981) *Cholesterol Autoxidation,* Plenum Press, New York, NY, London.
31. Skrede, B., Björkhem, I., Bergesen, Ø., Kayden, H.J., and Skrede, S. (1985) *Biochim. Biophys. Acta 836,* 368-375.
32. Eichenberger, W., and Grob, E.C. (1970) *FEBS Lett. 11,* 177-180.
33. Eichenberger, W. (1977) in *Lipids and Lipid Polymers in Higher Plants* (Tevini, M., and Lichtenthaler, H.K., eds.) pp. 169-182, Springer-Verlag, Heidelberg, New York, NY.
34. Young, N.L., Lopez, D.R., McNamara, D.J., and Benavides, G. (1985) *J. Lipid Res. 26,* 62-69.
35. Rujanavech, C., and Silbert, D.F. (1986) *J. Biol. Chem. 261,* 7251-7259.
36. Child, P., and Kuksis, A. (1986) *Biochem. Cell Biol. 64,* 847-853.
37. Nair, P.P., Turjman, N., Kessie, G., Calkins, B., Goodman, G.T., Davidovitz, H., and Nimmagadda, G. (1984) *Am. J. Clin. Nutr. 40,* 927-930.
38. Karcher, G. (1982) *Med. Welt 33,* 1763-1766.
39. Kuroki, S., Muramoto, S., Kuramoto, T., and Hoshita, T. (1983) *J. Pharmacobiodyn. 6,* 551-557.
40. Prasad, V.V., El-Maraghy, E., Ponticorvo, L., and Lieberman, S. (1985) *Proc. Natl. Acad. Sci. 82,* 2657-2659.
41. Ulloa, N., and Nervi, F. (1985) *Biochim. Biophys. Acta 837,* 181-189.
42. Kawamata, M., Ushimaru, H., Sano, A., and Takahashi, Y. (1975) Ger. Patent 2,458,890.
43. Mironova, V.N., and Kalashnikova, L.A. (1982) *Farmakol. Toksikol. (Moscow) 45,* 45-47.
44. Chattopadhyay, S., Chaudhuri, S., and Ghosal, S. (1987) *Planta Med. 1987,* 16-18.
45. Malinow, M.R., McLaughlin, P., and Stafford, C. (1978) *Am. J. Clin. Nutr. 31,* 814-818.
46. Malinow, M.R., Elliott, W.H., McLaughlin, P., and Upson, B. (1987) *J. Lipid Res. 28,* 1-9.

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Genetic Variations in Serum Lipid Levels of Inbred Mice and Response to Hypercholesterolemic Diet

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The serum lipid contents of a number of inbred and congenic strains of mice were measured. There were interstrain variations in each of the lipid fractions in mice fed a normal diet. Male and female C3H mice had the highest total cholesterol level; AKR mice showed the lowest values. Serum phospholipids were correlated well with cholesterolemia. The greatest variations between strains were in the triglyceride levels. There also was significant variation in the high density lipoprotein cholesterol serum levels (from 73–88% of the total cholesterol). The response to a hypercholesterolemic diet (1% cholesterol) was tested in seven inbred strains. All strains showed changes in serum cholesterol and in the proportions of the lipoproteins fractions. There was a large increase in the low density lipoprotein + very low density lipoprotein fractions. Feeding the diet revealed marked interstrain differences in the responses of the serum cholesterol and electrophoretic lipoprotein profiles. The C57BL/6 and B10.D2 strains were hyperresponders to the hypercholesterolemic diet with 71% and 63% of their serum cholesterol in the low density lipoprotein plus very low density lipoprotein fractions, respectively.
Lipids 23, 48–54 (1988).

Epidemiological studies in humans have demonstrated an association between elevated levels of serum cholesterol and risk of atherosclerosis. The major factors controlling cholesterol levels in man have been studied; environmental and particularly dietary factors were shown to be significant but during the last few years the importance of genetic factors has become increasingly apparent.

Normocholesterolemic rats and mice probably are inappropriate experimental animals because, contrary to humans, cholesterol is mainly carried in them in high density lipoproteins (HDL) and to a lesser extent by a fraction associated with low density lipoproteins (LDL) (1). However, the availability of inbred strains of mice has provided a number of new alternatives. A variety of inbred strains of mice currently are available for genetic experimentation.

The blood cholesterol levels of different strains of mice have been reported by Bruell et al. (2) and Yamamoto et al. (3). They also showed sex-dependent differences; the cholesterol level was higher in males than in females.

The pattern of inheritance governing the blood cholesterol level has been demonstrated by Bruell (4) and Yamamoto et al. (3) in inbred strains and by Weibust (5) by selecting high and low cholesterol lines on a normal low fat, low cholesterol diet.

Abbreivations: ASAT, abdominal subcutaneous adipose tissue; EAT, epididymal adipose tissue; HDL, high density lipoproteins; HDL-C, high density lipoprotein cholesterol; LDL, low density lipoproteins; PAT, perirenal adipose tissue; VLDL, very low density lipoproteins.

Dietary factors, particularly a high fat and high cholesterol diet, were found to produce atheromatous lesions in C57BL/6J inbred mice (6).

Inbred strains exhibited marked differences in their reaction to a high fat and high cholesterol diet. The C57BR/CdJ strain appeared to be particularly susceptible, whereas CBA/J mice proved to be resistant to atherosclerosis (7); this work examined the relationship between the serum total cholesterol level and the size of atheromatous lesions in the wall of the aortic sinus.

However, recent studies have shown that the evaluation of coronary risk by total serum cholesterol levels alone was too restrictive. Triglyceride, LDL and apoprotein (Apo-B) also are risk factors while HDL and Apo-AI appear to have a protective role against coronary heart disease.

However, C57BR mice are not available in France, and no quantitative data are available on the values of circulating lipids in the males and females of inbred strains of mice. Therefore, the present study was undertaken to provide these parameters for those strains that are available in France.

The second experiment was performed to determine the lipoprotein profile in males of seven inbred strains and their susceptibility to hypercholesterolemic diet.

MATERIALS AND METHODS

Animals and diets. In experiment 1, ten male and 10 female mice from each of 11 different inbred and two congenic strains were obtained from three different colonies (Table 1).

TABLE 1

Inbred and Congenic Strains of Mice from Different Vendors

Charles River, France	CSEAL-CNRS	IFFA-CREDO
Inbred strains		
C57BL/6Cr IBR	C57BL/6 JOrl	C57BL/6 JIco
C3H/He Cr IBR	C3H/He JOrl	
	C3H/eB JOrl	
		C3H/Ico
		C3H/ou JIco
DBA/2 Cr IBR	DBA/2 JOrl	DBA/2 JIco
BALB/c Cr IBR	BALB/c JOrl	BALB/cBy JIco
	CBA/JOrl	CBA/JIco
	A/JOrl	
	AKR/JOrl	
		B6D2F1/JIcO
Congenic strains	A.TH/Orl	
	A.TL/Orl	

Animals were received at four wk of age and acclimated for one wk before the study began. Males and females were housed separately in sibling groups of five animals in polypropylene cages. The temperature (22 ± 1 C), relative humidity (50 ± 10%) and light cycle (12 hr dark/light) of the room were controlled. All mice had free access to water and a normal chow diet (Table 2).

In experiment 2, male mice (4 wk old) of the inbred strains AKR/JOrl, BALB/cJOrl, CBA/JOrl, C3H/HeJOrl, C57BL/6JOrl, DBA/2JOrl, B10.D2Orl were obtained from the CSEAL-CNRS, Orléans, France. Mice were fed ad libitum normal diet (N) (Table 2) and water.

After one week, mice were matched for weight and half of them fed a hypercholesterolemic diet (C) formulated in our laboratory (Table 2). The mice were weighed weekly and killed after they had been on the experimental diet for four weeks. Food was withdrawn four hr before the mice were killed.

Animals were bled from the retroorbital venous sinus between 10–12 a.m., and blood was alllowed to clot for two hr at ambient temperature before centrifugation at 4500 tr/min for 20 min to obtain serum.

In experiment 2, the blood from three mice were pooled and clotted. Sera were drawn and aliquoted into tubes chilled on ice. Analyses were made on fresh sera within 48 hr after sacrifice. The heart, spleen, kidney and gonad were removed and weighed, as were the epididymal (EAT), perirenal (PAT) and abdominal subcutaneous (ASAT) adipose tissues.

Chemical analyses. Triglycerides were assayed using a Boehringer-Mannheim kit based on the enzymatic method developed by Wahlefeld (9). Phospholipids were assayed with the Wako phospholipid B Test kit (Biolyon, 69570, Dardilly, France). Total and free cholesterol levels were assayed by the enzymatic method of Röschlau et al. (10) with Boehringer-Mannheim kits. Esterified cholesterol was calculated by multiplying the difference between total and free cholesterol by 1.67; this figure corresponds to the ratio of the average molecular weight of human cholesteryl ester to that of free cholesterol.

Serum high density lipoprotein cholesterol (HDL-C) was measured in the supernate obtained after precipitation of very low density lipoproteins (VLDL) and LDL with phosphotungstic acid/MgC12 (Bio Merieux kit no. 61531) by the method of Burnstein et al. (11).

Lipoproteins were separated by agarose gel electrophoresis using agarose universal electrophoresis film (Corning Medical and Scientific, Palo Alto, CA). The buffer was 0.05 M barbital (Universal PHAB Corning).

After electrophoresis for 35 min at 90 v, the film was dried and then stained with Fat Red (Corning).

Lipoproteins also were separated by polyacrylamide gel electrophoresis. Five μl samples of serum were prestained with Sudan Black (v/v) and then electrophoresed on polyacrylamide gel slabs (Lipofilm Sebia) for one hr at 8 mA/slab.

Statistical analysis. In experiment 1, all results were analyzed by Tukey's test (12). The means were ranked and adjacent means were tested using the Student's t-test at an unadjusted significant level of 5%. Any groups of results separated by this percentage then were treated separately, the group mean was calculated and the greatest difference from all means of the group obtained. Finally, the individual variance ratio was estimated. All groups separated by this method are significantly different ($P < 0.05$). A Duncan's test (13) was used for statistical evaluation of sex differences.

In experiment 2, all results are expressed as mean ± SEM. Statistical differences between strains and diets were estimated by Duncan's test (13).

TABLE 2

Composition of Normal Diet (N) and Hypercholesterolemic Diet (C)

	g/100 g	
	N	C
Cellulose	3.7	5.0
Yeast	4.0	4.0
Corn oil	2.7	2.0
DL methionine	0.46	0.4
Mineral mixture[a]	3.5	3.5
Vitamin mixture[b]	2.2	2.2
Cow's milk casein	10.0	21.0
Wheat flour	73.44	0
Sucrose	0	49.4
Coconut oil	0	11.0
Cholesterol	0	1.0
Cholate	0	0.5
	%	%
Proteins	17.9	22.4
Lipids	3.9	13.5
Carbohydrates	55.1	50.6
Digestible energy per 100 g	1.36MJ	1.73MJ

[a,b]Mineral mixture and vitamin mixture as described elsewhere (8).

RESULTS

Experiment 1. Body weights. The total range body weights between strains at nine wk of age was small in males (21.6–27.8 g) and in females (18.44–24.1 g). The growth of the mice was identical in all strains. The differences observed in body weights between strains at the end of the experiment were due to differences at weaning, with the daily weight increases being similar in all strains.

Serum cholesterol levels. The distribution of total cholesterol varied with strain in male mice (Fig. 1). Total cholesterol levels were highest in the C3H strains and lowest in the AKR mice, irrespective of supplier. All female C3H mice, irrespective of supplier and substrain, had total cholesterol levels, which placed them in the same group with high levels of cholesterol. In males, the C3H mice were ranked into two groups and showed the highest values. Conversely, AKR mice had a low level of total cholesterol. For free cholesterol, all females were ranked in the same group even though the range of values was 16.1 for AKR and 30.1 mg/dl for C3H/ouJIco. The values for males varied from 42 mg/dl for DBA/2CrIBR to 15.1 mg/dl for AKR. Differences in esterified cholesterol levels accounted for the increased values of total cholesterol in C3H strains. There was no sex-dependent difference in the serum cholesterol of five strains (C57BL/6CrIBR, C3H/HeCrIBR, C3H/HeJOrl, BALB/cJOrl, AKR/JOrl).

Serum triglyceride levels. There were great variations in serum triglyceride concentrations of male mice between

strains; DBA/2 male mice were hypertriglyceridemic (300 mg/dl for DBA/2JOrl, 303 mg/dl for DBA/2JIco, and 375 mg/dl for DBA/2CrIBR) compared to 97 mg/dl for AKR mice. The differences between strains were smaller in females (254 mg/dl for DBA/2JIco vs 99 mg/dl for C57B//CrIBR), and only two groups were separated by Tukey's test. Comparisons of male and female means within each strain showed contradictory results. In two strains, the female means were higher than those of the males (B6D2F1/JIco and C3H/HeCrIBR). No difference between sexes was observed in any of the C3H and BALB strains or in the DBA/2JIco, CBA/JOrl and C57BL/6CrIBR strains. In the other strains, male mice had significantly higher serum triglyceride levels than did the females. As a result, the distribution of serum triglyceride levels was identical in males and females.

Serum phospholipid levels. In male mice, four groups were separated by statistical analysis. Levels varied from 184 mg/dl in male AKR mice to 31 mg/dl in male C3H/He mice. Except in four strains (C3H/HeCrIBR, BALB/cJOrl, AKR/JOrl and C57BL/6JIco), the means for males consistently were higher than those of females. In females, only two groups were identified. Serum phospholipids were correlated well (r = 0.859) with serum total cholesterol.

Experiment 2. Body and organ weights. Regular growth curves were observed in all the strains fed the normal diet. Body weight was unaffected by the hypercholesterolemic diet. The significant differences between the strains were due solely to the specific body weight of each strain at weaning. Differences in organ weights observed between the strains were related to the normal weight variations regardless of the type of diet.

Serum parameters. Figures 2 and 3 show the serum total cholesterol, triglyceride, and phospholipid levels of the different strains of mice maintained on the normal diet (N) and the hypercholesterolemic diet (C). On normal diet, the C3H/He mouse strain was hypercholesterolemic and hyperphospholipidemic. The AKR mice were hypocholesterolemic and the DBA/2 mice were hypertriglyceridemic.

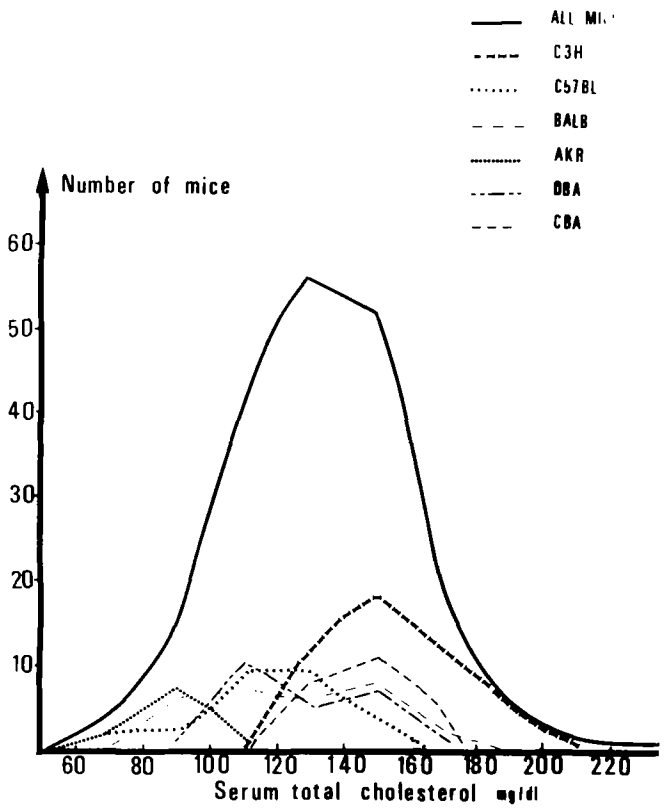

FIG. 1. Distribution of total cholesterol levels in male mice of different inbred and congenic strains.

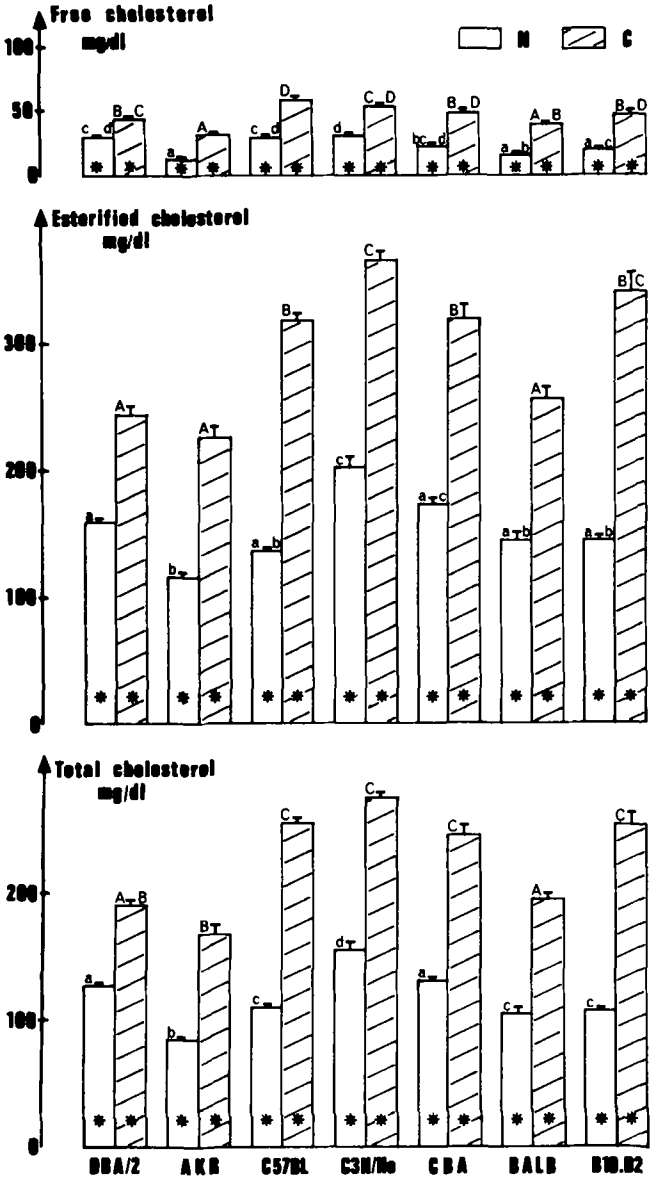

FIG. 2. Total, esterified and free cholesterol concentrations in the serum of male inbred mouse strains fed either a normal diet (N) or a hypercholesterolemic diet (C). Mice were killed in a fasted state at nine weeks of age. Each bar represents the mean and SEM of data from 10 pools of three mice, except for B10.D2 (5 pools). For comparison between strains, on the diet N, bars not sharing a common small letter are significantly different $p < 0.05$; on diet C, bars not sharing a common capital letter are significantly different $p < 0.05$. For comparison between diets for one strain, *, a significant difference $p \leq 0.05$; **, a highly significant difference $p \leq 0.01$.

On diet C, all the strains exhibited very significantly higher levels of free, esterified and total cholesterol but the increases differed from strain to strain. Serum cholesterol levels were more than doubled in C57BL/6 and B10.D2 strains; the lowest increase was observed in DBA/2 strain. The C3H/He mice had the highest cholesterol level when fed diet N, whereas on diet C this parameter was not different significantly from those of the other strains. The strains fed diet C were divided into two groups: similar high cholesterol levels (>250 mg/dl) in C57BL/6, C3H/He, CBA and B10.D2 strains; lowest cholesterol levels in DBA/2, AKR, and BALB/c strains (<200 mg/dl).

Administration of diet C induced a very significant increase in the phospholipid levels only in AKR and BALB/c strains; in contrast, the triglyceride levels decreased significantly except in the BALB/c and B10.D2 mice. Table 3 gives the distribution of cholesterol in the lipoproteins. The high density lipoproteins carried ca. 80% of the serum cholesterol in all strains fed the normal diet. In C3H mice, the HDL cholesterol level was different significantly from that of all other strains except CBA mice, with these two strains showing the highest values.

DBA/2 and C57BL/6 mice had greater cholesterol concentrations in the LDL and VLDL fractions. Cholesterol feeding promoted a rise in the HDL-C level except in C57BL/6, BALB/c and B10.D2 strains. But the HDL cholesterol/total cholesterol ratio dropped markedly in all strains after feeding the high cholesterol diet; the decrease was more pronounced in the C57BL/6 and B10.D2 strains. The level of (LDL + VLDL) cholesterol rose after cholesterol feeding in all strains. The most striking increase was observed in B10.D2 mice and the least increase in DBA/2 mice. On diet C, very significant differences were observed between strains; C57BL/6 and B10.D2 mice had the highest values.

On the normal diet, DBA/2 and C57BL/6 mice, had the largest (LDL + VLDL) cholesterol/total cholesterol ratio. Diet C induced a rise in this ratio in all strains except DBA2 mice; the highest ratio was observed in C57BL/6 and B10.D2 mice. The LDL + VLDL/HDL cholesterol ratio increased with cholesterol feeding; this ratio was very high in B10.D2 and C57BL/6 mice as compared to other strains.

Electrophoretic patterns of lipoproteins. On the normal diet, the sera of fasted mice exhibited a major α-band (HDL) on agarose gels. A pre-β band (VLDL) and a β-band (LDL) were seen in C57BL/6, B10.D2 and DBA/2 strains. This separation was more distinct on polyacrylamide gels. Administration of diet C induced a marked increase in the pre-β band masking the appearance of β-VLDL in all strains, with a greater increase in the C57BL/6 and B10.D2 strains.

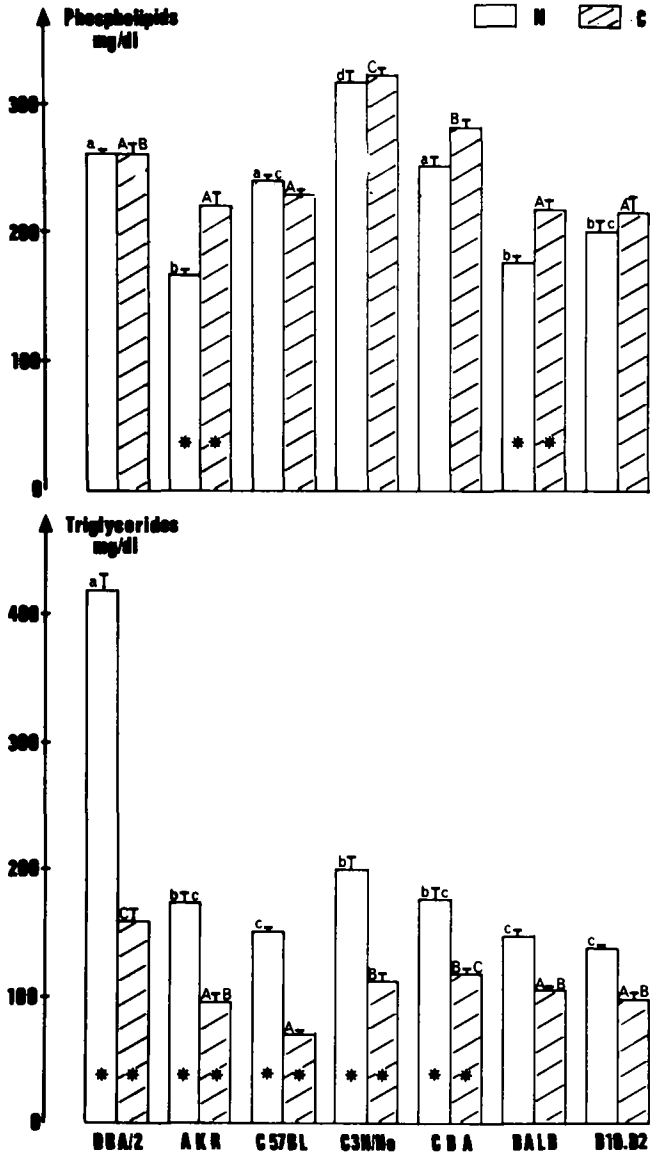

FIG. 3. Triglyceride and phospholipid concentrations in the serum of male inbred mouse strains fed either a normal diet (N) or a hypercholesterolemic diet (C). Animals were killed in a fasted state at nine wk of age. See Figure 2 for legends.

DISCUSSION

Additive inheritance of plasma cholesterol level in mice was established by Bruell (2,4) and Yamamoto et al. (3), using inbred strains and their hybrids. This was confirmed by Weibust (5) by selective breeding of mice with high or low cholesterol levels. The present study demonstrates that all serum lipid levels vary with the strain in mice. Bruell (2,4) and Weibust (5) observed significant sex differences in serum cholesterol level in inbred strains, hybrids and selected lines. In this study and as observed by Yamamoto et al. (3) in the STR/1N strain, the levels of circulating lipids in males were not higher than in females in all strains. Differences in the age of the mice could not be invoked to explain the differences between this study and other studies (2,4,5) because previous investigations have indicated that serum cholesterol levels do not vary with age in mice (2,3) in contrast with observations in humans (14).

A control diet was used to determine whether there were genetic differences between the circulating lipids of a great number of inbred mouse strains of both sexes. The greatest variations among strains was in their triglyceride levels. In males, the mean was increased by 285% in the hypertriglyceridemic strain (DBA/2CrIBR) as compared

TABLE 3

Lipoprotein Cholesterol Levels (mean ± SEM) in Different Strains of Mice Fed a Normal Diet (N) or an Hypercholesterolemic Diet (C)

		DBA/2	AKR	C57BL/6	C3H/He	CBA	BALB/c	B10.D2
HDL C mg/dl	N	92.5 ±1.48^{a} } **	67.1 ±3.44^{b} } **	81.8 ±4.04^{ab}	128.7 ±5.92^{c} } *	111.6 ±3.85^{cd} } *	91.8 ±3.22^{ad}	90.6 ±1.96^{abd}
	C	118.5 ±3.98^{bc}	95.1 ±3.69^{d}	74.1 ±1.60^{e}	153.9 ±4.57^{a}	136.3 ±3.49^{ab}	105.4 ±2.72^{cd}	92.6 ±3.50^{de}
LDL+VLDL C mg/dl	N	34.0 ±1.73^{d} } **	15.8 ±2.18^{ab} } **	29.3 ±4.07^{cd} } **	25.1 ±3.73^{bcd} } **	16.3 ±2.78^{ab} } **	12.1 ±2.16^{a} } **	17.4 ±1.57^{abc} } **
	C	72.5 ±4.60^{a}	73.3 ±5.34^{a}	179.5 ±5.49^{f}	129.9 ±4.71^{d}	108.1 ±5.49^{c}	89.8 ±6.05^{b}	160.2 ±10.13^{e}
HDL C / CT	N	0.73±0.011^{a} } *	0.82±0.026^{ab} } **	0.77±0.023^{ac} } **	0.84±0.023^{ab} } **	0.87±0.022^{bc} } **	0.88±0.021^{b} } **	0.84±0.014^{abc} } **
	C	0.62±0.021^{a}	0.57±0.022^{a}	0.30±0.009^{b}	0.56±0.015^{a}	0.56±0.014^{a}	0.54±0.021^{a}	0.37±0.017^{b}
LDL+VLDL C / CT	N	0.30±0.028^{c}	0.19±0.029^{ab} } **	0.26±0.035^{bc} } **	0.16±0.023^{a} } **	0.13±0.022^{a} } **	0.11±0.021^{a} } **	0.16±0.014^{a} } **
	C	0.38±0.021^{a}	0.43±0.022^{a}	0.71±0.011^{b}	0.44±0.015	0.44±0.014^{a}	0.46±0.021^{a}	0.63±0.017^{b}
LDL+VLDL C / HDL C	N	0.37±0.021^{bc} } **	0.25±0.050^{abc} } **	0.41±0.095^{c} } **	0.21±0.037^{ab} } **	0.15±0.031^{a} } **	0.14±0.026^{a} } **	0.24±0.060^{abc} } **
	C	0.63±0.053^{a}	0.79±0.076^{b}	2.43±0.128^{d}	0.76±0.057^{ab}	0.79±0.049^{b}	0.87±0.081^{b}	1.70±0.106^{c}

Values in the same line not sharing the same common superscript letter are highly significantly different ($P \leq 0.01$). Effect of hypercholesterolemic diet: *, significantly ($P \leq 0.05$), **, highly significantly ($P \leq 0.01$) different from normal diet.

to AKR mice, and in females by 193% in DBA/2JIco as compared to C57BL/6JOrl.

Thompson (6) and Paigen et al. (15) have stated that C57BL/6 mice can be defined as susceptible to atheromata. In our study and as observed by Morrisett et al. (16), the susceptible strain of mice consistently maintained a lower triglyceride level on the control diet than did resistant mice (CBA) in both sexes.

Variations in phospholipid level between strains were lower than for triglyceride and more elevated for males than for females (71% increase for male C3H/HeJOrl vs AKR, 46% increase for female C3H/HeCrIBR vs DBA/2JIco). As observed by Bruell et al. (2) and Paigen et al. (15), the highest values of serum cholesterol were found in both sexes of C3H mice, whatever the substrain. In contrast, AKR male mice showed the lowest values not only for choiesterol but for all circulating lipids. The variations were less pronounced in females. If we compare our results with those of Morrisett et al. (16) and Breckenridge et al. (17), we observe that the susceptible strain of mice maintained a somewhat lower level of serum cholesterol than did the resistant strain on control diet. In contrast to their results, we found an average serum cholesterol of 117 mg/dl in C57BL/6 mice and 142 mg/dl in CBA mice. This could be in agreement with the experiment of Paigen (15) who observed that there is a low correlation between cholesterol levels and lesion formation on atherogenic diet.

In contrast to results reported by Breckenridge et al. (17) indicating that the susceptible strain of mice (C57BR) gained less weight than the resistant one (CBA), all the inbred lines showed similar growth rates in our second experiment, and the differences in body weight at weaning accounted for variations in weight observed at the end of the experiment. While differences in organ weights were observed between strains, the organ/body weight ratios were similar in all strains, except for adipose tissues where two strains of mice, DBA/2 and CBA, seem to be fatter than the others, regardless of the diet consumed. These results confirm earlier observations (18–20) of differences in fat accumulation by different mouse strains, an effect related to inefficient food utilization by the strains with lower ability to store energy as fat (21).

Morbidity and mortality were not observed in response to a high cholesterol diet, as in previous studies (6,7). The diet was tolerated well by all strains, and body weight was unaffected by diet at nine weeks of age after four weeks of ad libitum administration, as observed previously in rats (22). There is now little doubt that the quantity and type of fatty acids in the diet profoundly can influence the level of circulating cholesterol in most species, including man. In the C57BR/cdJ mouse, serum total cholesterol responded to change in P/S ratio only when cholesterol was added to the diet (23); this also is observed in man (24). Most of the apolipoproteins seem to increase when high saturated fat diets are consumed (24–26). A high carbohydrate consumption leads to high plasma cholesterol in the mouse (27).

Feeding our hypercholesterolemic diet, which is rich in saturated fatty acids, carbohydrate and cholesterol, to mice resulted in an increase of about 70–140 mg of cholesterol over that of animals maintained on the normal diet. The cholesterol levels measured in any of the strains were not as high as the level observed by Roberts and Thompson (7) in C57BR strain. This may be due to strain differences or to our diet being moderately enriched in cholesterol. Our results are more similar to those obtained by Paigen et al. (15) who examined female mice of different inbred lines fed an atherogenic diet containing 1.25% cholesterol. However, in this study, DBA/2 mice had the lowest increase in serum cholesterol level, whereas Paigen et al. (15) found that this strain had the highest one. The main effect induced by hypercholesterolemic diet was observed in the C57BL/6 and

B10.D2 strains in which serum cholesterol levels were more than doubled. CBA mice have been described as being resistant to diet-induced elevation of serum cholesterol. When fed a high cholesterol diet (7), this strain could not be differentiated from the other strains in this experiment, mean values were increased by 93%. In rats, the effect of feeding a cholesterol-enriched diet upon serum triglyceride levels led to contradictory results (22,28,29). In this study, the enriched cholesterol diet induced a decrease in serum triglyceride levels in all strains, the main effect being observed in the hypertriglyceridemic strain (DBA/2), which had a decrease of 62%.

Cholesterol feeding reduces plasma triglycerides, whereas it increases the liver triglycerides (30). The changes probably are due to a shift of hepatic lipoprotein secretory mechanisms for triglyceride-rich particles of lipoprotein towards cholesteryl esters-enriched particles in response to excess cholesterol deposition in the liver.

Recent studies demonstrate that elevated LDL levels are correlated positively with coronary heart disease in humans (31), whereas HDL appear to protect against atherosclerosis disease (32-34). Genetic variations in the structure and quantities of lipoproteins and structural variations of apolipoproteins have been demonstrated in various mouse strains (16,17,35-37). In this study, 70-80% of the cholesterol is associated with HDL, which is in agreement with results obtained by other investigators with different strains, using ultracentrifugal procedures (37-39). On the normal diet, the HDL of C3H/He and CBA mice displayed an elevated cholesterol content; in contrast, in the hypertriglyceridemic DBA/2 mice the HDL fraction had a lower cholesterol content compared to other strains. An inverse correlation between HDL cholesterol and the level of serum triglyceride has been demonstrated in humans (40).

The administration of cholesterol-enriched diet to rats induced a rise in lipoproteins corresponding to β-VLDL (41). In this study, the electrophoretic profiles of lipoproteins from mice fed a cholesterol-rich diet indicate an increase of VLDL fraction in all strains. This is in agreement with the precipitating method where a dramatic increase in LDL + VLDL cholesterol was observed in all strains fed diet C (× 2 to 9 in DBA/2 and B10.D2, respectively). It is remarkable that 38 (DBA/2) to 71% (C57BL/6) of the serum cholesterol was found in the LDL + VLDL fraction. More detailed studies are needed to establish which of the two fractions is more affected. Terpstra and Beynen (39) in Swiss mice and Tsuda et al. (42) in ICR mice noted a rise in HDL cholesterol during administration of an atherogenic diet. In our experiment, mice could be divided into two groups: HDL cholesterol increased in DBA/2, AKR, C3H/He and CBA, whereas this parameter was not altered for the other strains.

The (LDL-C + VLDL-C) to HDL-C ratio was increased markedly in all the animals fed the hypercholesterolemic diet because of the large effect of diet C on the other lipoprotein fraction.

These results indicate that the C57BL/6 and the B10.D2 mice are very susceptible to hypercholesterolemic diet but no resistant strain could be identified. The availability of inbred strains of mice with different cholesterolemic responses to change in diet may be of importance. As demonstrated by Paigen et al. (15) and Morrisett et al. (16), atherosclerotic lesions can be produced experimentally in inbred mice, and these mouse strains may prove useful in studying genetic influences on the development of atherosclerosis.

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REFERENCES

1. Day, C.E., Phillips, W.A., and Schurr, P.E. (1979) *Artery 5,* 90-109.
2. Bruell, J.H., Daroczy, A.F., and Hellerstein, H.K. (1962) *Science 135,* 1071-1072.
3. Yamamoto, R.S., Crittenden, L.B., Sokoloff, L., and Jay, G.E. (1963) *J. Lipid Res. 4,* 413-418.
4. Bruell, J.H. (1963) *Science 142,* 1664-1665.
5. Weibust, R.S. (1973) *Genetics 73,* 303-312.
6. Thompson, J.S. (1969) *J. Atheroscler. Res. 10,* 113-122.
7. Roberts, A., and Thompson, J.S. (1976) *Adv. Exp. Med. Biol. 67,* 313-326.
8. Aubert, R., Suquet, J.-P., and Lemonnier, D. (1980) *J. Nutr. 110,* 649-661.
9. Wahlefeld, A.W. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.U., ed.), pp. 1831-1835, Academic Press, New York, NY.
10. Röschlau, P., Bernt, E., and Gruber, W. (1974) *Z. Klin. Chem. Klin. Biochem. 12,* 403-407.
11. Burnstein, M., Scholnick, H.R., and Morfin, R. (1970) *J. Lipid Res. 11,* 583-598.
12. Tukey, J.W. (1954) in *Statistical Analysis in Chemistry and the Chemical Industry* (Bennett, C.A., and Franklin, N.L., eds.), pp. 339-344, John Wiley, New York, NY.
13. Duncan, D.B. (1968) in *Computational Handbook of Statistics* (Brunning, J.L., and Kintz, B.L., eds.) pp. 115-117, Scott, Foresman and Company, Glenview, IL.
14. Schilling, F.J., Christakis, G.J., Benett, N.J., and Coyle, J.F. (1964) *Am. J. Public Health 10,* 113-122.
15. Paigen, B., Morrow, A., Brandon, C., Mitchell, D., and Holmes, P. (1985) *Atherosclerosis 57,* 65-73.
16. Morrisett, J.D., Kim, H-S., Patsch, J.R., Datta, S.K., and Trentin, J.J. (1982) *Arteriosclerosis 2,* 312-324.
17. Breckenridge, W.C., Roberts, A., and Kuksis, A. (1985) *Arteriosclerosis 5,* 256-264.
18. Fenton, P.F., and Carr, C.J. (1951) *J. Nutr. 45,* 225-233.
19. Lemonnier, D. (1972) *J. Clin. Invest. 51,* 2907-2915.
20. Hoover-Plow, J.L. (1984) *Nutr. Rep. Int. 29,* 1171-1179.
21. Hoover-Plow, J.L., and Nelson, B. (1985) *J. Nutr. 11,* 303-310.
22. Hulbron, G., Aubert, R., Bourgeois, F., and Lemonnier, D. (1982) *J. Nutr. 112,* 1296-1305.
23. Mulvihill, B.J., and Walker, B.L. (1984) *Nutr. Res. 4,* 611-612.
24. Shepherd, J., Packard, C.J., Patsch, J.R., Gotto, A.M., and Tauton, O.D. (1978) *J. Clin. Invest. 61,* 1582-1592.
25. Shepherd, J., Packard, C.J., Grundy, S.M., Yeshurun, D., Gotto, A.M., and Taunton, O.D. (1980) *J. Lipid Res. 21,* 91-99.
26. Nicholosi, R.J., Marlett, J.A., Morello, A.M., Flanagan, S.A., and Hegsted, D.M. (1981) *Atherosclerosis 38,* 359-371.
27. Davies, R.H., Kramer, D.L., and Sackman, J.W. (1974) *Lancet i,* 750-752.
28. Mahley, R.W., and Holcombe, K.S. (1977) *J. Lipid Res. 18,* 314-324.
29. Mengheri, E., Scarino, M.L., Vignolini, F., and Spadoni, M.A. (1985) *Br. J. Nutr. 53,* 223-232.
30. Guo, L.S.S., Hamilton, R.L., Ostwald, R., and Havel, R.J. (1982) *J. Lipid Res. 23,* 543-555.
31. Frederickson, D.S., and Levy, R.I. (1972) in *The Metabolic Basis of Inherited Disease* (Stanbury, J.B., Wyngaarden, J.B., and Frederickson, D.S., eds.), pp. 545-614, McGraw-Hill, New York, NY.

32. Miller, G.J., and Miller, N.E. (1975) *Lancet i,* 16-19.
33. Gordon, T., Castelli, W.P., Hjortland, M.C., Kannel, W.B., and Dawber, T.R. (1977) *Am. J. Med. 62,* 707-714.
34. Assman, G., and Schriewer, H. (1980) *Nutr. Metab. 24* (suppl. 1), 19-25.
35. LeBoeuf, R.C., Puppione, D.L., Schumaker, V.N., and Lusis, A.J. (1983) *J. Biol. Chem. 258,* 5063-5070.
36. Lusis, A.J., Taylor, B.A., Wangenstein, R.W., and LeBoeuf, R.C. (1983) *J. Biol. Chem. 258,* 5071-5078.
37. Camus, M.C., Chapman, M.J., Forgez, P., and Laplaud, P.M. (1983) *J. Lipid Res. 24,* 1210-1228.
38. Hsu, K.H.L., Ghanta, V.K., and Hiramoto, R.N. (1981) *Immunology 126,* 1909-1913.
39. Terpstra, A.H.M., and Beynen, A.C. (1984) *Comp. Biochem. Physiol. 77B,* 523-528.
40. Castelli, W.P., Doyle, J.T., Gordon, T., Hames, C.G., Hjortland, M.C., Hully, S.B., Kagan, A., and Zukel, W.J. (1977) *Circulation 55,* 767-772.
41. Mahley, R.W. (1978) in *Disturbances in Lipid and Lipoprotein Metabolism* (Dietschy, J.M., Gotto, A.M., and Ontko, J.A., eds.), pp. 181-197, American Physiological Society, Bethesda, MD.
42. Tsuda, M., Kitazaki, T., and Imai, Y. (1983) *J. Biochem. 93,* 1071-1077.

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Subcellular Distribution of Disaturated Phosphatidylcholine in Developing Rabbit Lung

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To determine the subcellular distribution of disaturated phosphatidylcholine (DSPC) in lung tissue during perinatal development, fetal rabbits at 24, 26, 28 and 31 (term) days gestation and newborns were studied. Following alveolar lavage, fractions enriched in nuclei-cellular debris, mitochondria, microsomes, surfactant (lamellar bodies) and cytosol were prepared from the residual tissue homogenate, and their DSPC content was determined. The DSPC content of the unfractionated residual lung tissue homogenate progressively and significantly increased during fetal development, rising from 9.09 ± 0.91 to 17.45 ± 2.88 mg/g dry lung between 24 days gestation, and term. Between 24 and 26 days gestation the overall increase in tissue DSPC was due to a two-fold increase in the mitochondrial, microsomal and cytosolic pools. Lamellar bodies were first isolable at 26 days gestation. The DSPC content of this fraction increased six-fold (from 0.10 ± 0.02 to 0.67 ± 0.15 mg/g dry lung) between 26 and 28 days gestation and a further seven-fold (to 4.63 ± 1.06 mg/g dry lung) by term, accounting for the overall increase in the tissue homogenate value during this time period. By the first postnatal day, microsomal and cytosolic DSPC increased another two-fold, but no significant change occurred in the other subcellular fractions. Alveolar lavage DSPC progressively increased over the time period studied. While there was no change in the lamellar body DSPC/total PC ratio during fetal development, each of the mitochondrial, microsomal and cytosolic ratios decreased between days 26 and 28 of gestation and then increased at term. Our results indicate that in addition to the pulmonary surfactant, for which DSPC is often used as a marker, other subcellular organelles contain significant DSPC pools that undergo dynamic changes in size during perinatal development.
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Lung surfactant, the phospholipid-rich material that lines the mammalian lung and prevents atelactasis by reducing the surface tension at the air-alveolar interface (1), is produced by the fetal lung in increased quantity toward the end of gestation (2,3). Surfactant is synthesized in the Type II alveolar cell and stored in intracellular lamellar inclusion bodies prior to release to alveoli (4).

Phosphatidylcholine represents the major surfactant constituent (5,6), and the disaturated species, dipalmitoyl phosphatidylcholine, is the major component responsible for its surface tension-reducing properties (1,5,6). As the lung is particularly enriched in this phospholipid, its measurement in whole lung tissue is often used as a marker for the surfactant complex (7–10). It has been shown, however, that the disaturated species of DSPC is not uniquely localized in the surfactant complex (11–14). For example, in the adult, less than half of the total lung DSPC has been identified as being surfactant-associated (12,13) and even lower values have been found for fetal and newborn lung (15). The subcellular locale of the nonsurfactant DSPC has never been fully explored. Such a study would be of particular interest to pursue over the period of time in which the surfactant pool sizes undergo dramatic changes, i.e., during late fetal and early postnatal development (2,3). In our laboratory, we have recently developed methods for the quantitative isolation of surfactant pools from fetal and newborn rabbit lung (16). In the present study, we report on the distribution of lung DSPC over these and other subcellular fractions obtained during this time period.

MATERIALS AND METHODS

New Zealand white rabbits whose time of conception was known to within 1 hr were purchased from Rieman's Fur Ranch, St. Agatha, Ontario. The does were anesthetized with intravenous sodium pentobarbital, and the fetuses were delivered by cesarean section and immediately killed by intraperitoneal injection of sodium pentobarbital. Spontaneously delivered newborns were similarly killed in their first postnatal day.

Lungs were lavaged in situ with a total of seven 0.5–1.5 ml aliquots of 0.85% NaCl. We have previously shown that this technique recovers better than 90% of the total phospholipids recovered in 12 consecutive lavages (16). For each experiment, alveolar lavage returns obtained from either 5–8 fetuses or 2–3 newborns were pooled for lipid extraction and analysis as described below.

Following the lavage procedure, the residual lung tissue obtained in each experiment was pooled and finely minced, aliquots were removed for drying to constant weight and the remainder was fractionated as follows:

A 10% homogenate (w/v) in 0.01 M Tris buffer, containing 0.145 M NaCl and 0.001 M EDTA (pH 7.4), was prepared using a polytron homogenizer (Brinkman Instruments, Rexdale, Ontario) at a speed setting of 5.0 for 10–30 sec (in 10 sec bursts). Aliquots of

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Abbreviations: DSPC, disaturated phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DNA, deoxyribonucleic acid; UDP, uridine 5-diphosphate.

the tissue homogenate were removed for lipid extraction and analysis, and the remainder was centrifuged for 5 min at 140 × g. The resultant pellet, designated as the nuclear-cellular debris fraction, was saved for lipid extraction and analysis, while the resultant supernatant was further centrifuged for 30 min at 10,000 × g. The supernatant obtained from this centrifugation step was then centrifuged for 60 min at 100,000 × g to yield a microsomal-enriched pellet and cell cytosol fractions. Mitochondrial and lamellar body-enriched fractions were prepared from the 10,000 × g pellet material as follows: the pellet was suspended in the Tris buffer, and 2.0 ml aliquots were layered over discontinuous density gradients consisting of 5.0 ml each of 0.68 M and 0.25 M sucrose (prepared in the Tris buffer). The gradients were then centrifuged for 60 min at 65,000 × g in an SB-283 swinging bucket rotor (Damon/IEC Division, Needham Heights, MA), and the surfactant (lamellar body) fraction (which formed a discrete band at the interface between the sucrose layers) and the mitochondrial-enriched pellet were removed for lipid extraction and analysis. In a previous study in our laboratory in which we developed this technique for isolating surfactant from fetal and newborn lung tissue homogenates, we found better than 85% recovery of a pure radioactive-labeled lamellar body preparation added to homogenized lung. Of the 15% loss, 5% was present in the 140 × g pellet fraction, another 5% in the 10,000 × g supernatant and the remaining 5% spread over all the other fractions obtained in the isolation procedure. The details of this isolation scheme are described in a separate report (16).

Prior to lipid extraction and/or enzymatic analysis, all particulate fractions were washed once with 0.25 M sucrose. Lipids were extracted with chloroform/methanol (2:1, v/v) as previously described (17). Aliquots of the lipid extracts were removed for separation and analysis of the individual phospholipids by two-dimensional thin layer chromatography as described elsewhere (18). Further aliquots were removed, and the DSPC was isolated by column chromatography following osmium tetroxide oxidation as described by Mason et al. (19). The DSPC content of the recovered eluates was determined by phosphorus analysis (17). In some experiments, the purity of the isolated DSPC was assessed by phospholipid and fatty acid analysis of the recovered eluates according to previously described procedures (16). For each fraction and at each gestational age, the sample contained ca. 95% DSPC, of which almost 99% of the esterified fatty acids were saturated. Palmitic acid accounted for at least 80% of the total saturates. Corrections were not made for these values. For routine analysis, ^{14}C-dipalmitoylphosphatidylcholine (DPPC) containing 10,000 dpm was run through the column to assess the recovery in each individual run. At least 90% of the radioactivity was consistently recovered. In a preliminary study, we found that reproducible recoveries could be achieved in the range of 10–1000 μg DPPC.

Aliquots of each fraction were removed for analysis of the following marker enzymes: succinate dehydrogenase (EC 1.3.99.1) according to Pennington (20), NADPH-cytochrome c reductase (EC 1.6.2.4) as described by Omura and Takesue (21), lactate dehydrogenase (EC 1.1.1.27) using a commercial kit as described in Sigma Technical Bulletin No. 500 (Sigma Chemical Co., St. Louis, MO), 5′-nucleotidase (EC 3.1.3.5) according to Avruch and Wallach (22), UDP-galactose galactosyltransferase (EC 2.4.1.38) by the procedure of Beaufay et al. (23) as modified by Jobe et al. (24), and arylsulfatase (EC 3.1.6.1) as described by Chang et al. (25). At least one analysis was performed (in duplicate) on all the fractions obtained at each gestational age. Protein was determined according to Lowry et al. (26) and DNA as described by West et al. (27). A purified plasma membrane preparation was obtained by the method of Maeda et al. (28).

RESULTS

For each gestational age and in each experiment, ca. 70% of the total tissue homogenate DNA was recovered in the fraction designated as nuclear-cellular debris, indicating that the bulk of the cell nuclei were, in fact, recovered in this fraction. Marker enzyme analysis (Table 1) indicated the presence of each of the other subcellular organelles as well. Marker enzymes for mitochondria (succinate dehydrogenase), microsomes (NADPH cytochrome c reductase) and cytosol (lactate dehydrogenase) were appropriately enriched in the fractions so designated. Each of these fractions also contained some amount of plasma membranes, golgi and lysosomes, as evidenced by the presence of 5′-nucleotidase, UDP galactosyltransferase and arylsulfatase activities, respectively. The lamellar body fraction appeared to be reasonably devoid of contamination by other subcellular organelles. Using the data in Table 1, it can be estimated that this fraction is ca. 1% contaminated with mitochondria, 3.8% with microsomes and, using the value of 111.6 nmol/min/mg protein as the specific activity of a purified preparation of plasma membranes from adult lung tissue, 3.9% with this component. None of the other marker enzymes were analyzed on the isolated surfactant fractions.

The overall phospholipid composition was determined on each fraction at each gestational age. With the exception of total tissue homogenate and isolated lamellar bodies, there was little change in the relative proportion of total phosphatidylcholine over the development period studied. Tissue homogenate phosphatidylcholine increased from 51.1 ± 1.9% (n = 3) of total phospholipids on the 24th day of gestation to 57.4 ± 0.6% (n = 3) at term; it decreased slightly on the first postnatal day to 52.7 ± 1.7% (n = 3) and changed little thereafter. A lamellar body fraction was first isolable from the tissues on the 26th day of gestation. At this stage of development, phosphatidylcholine represented ca. 50% of the total lipid phosphorus. This value increased to almost 80% by the 28th day and changed little after that. Representative overall phospholipid compositions for each subcellular fraction, obtained from the fetus at term, are shown in Table 2. As indicated, phosphatidylcholine is the major phospholipid in each fraction but is particularly enriched in the alveolar lavage and isolated surfactant fractions.

TABLE 1

Representative Distribution of Marker Enzyme Activities in Rabbit Lung Subcellular Fractions

	Tissue homogenate	Nuclear-cellular debris	Mitochondrial-enriched	Lamellar body-enriched	Microsomal-enriched	Cytosol
			units/mg protein			
Succinate dehydrogenase[a]	2.4 ± 0.4	2.8 ± 0.4 (20.5)	49.4 ± 7.5 (68.6)	0.5 ± 0.2 (0.1)	3.4 ± 0.9 (0.7)	0.7 ± 0.0 (10.1)
NADPH-Cytochrome c reductase[a]	3.4 ± 0.4	4.8 ± 1.7 (17.5)	3.3 ± 0.4 (16.2)	1.2 ± 0.6 (0.5)	32.0 ± 9.2 (26.8)	1.6 ± 0.2 (38.8)
Lactate dehydrogenase[a]	332.7 ± 20.6	122.8 ± 5.4 (12.1)	36.7 ± 10.7 (4.4)	N.D.[b]	31.2 ± 5.9 (0.3)	552.7 ± 9.1 (83.2)
5′-Nucleotidase[a,c]	5.1 ± 0.8	6.1 ± 1.3 (23.3)	25.2 ± 2.3 (66.9)	4.3 ± 0.2 (0.7)	7.9 ± 1.7 (2.8)	0.9 ± 0.2 (6.3)
UDP galactosyltransferase[d]	3.0 ± 0.8	1.8 ± 0.1 (6.6)	14.3 ± 0.4 (42.8)	N.D.	3.8 ± 0.8 (30.3)	1.1 ± 0.8 (20.2)
Arylsulfatase[a]	0.15 ± 0.03	0.22 ± 0.03 (49.0)	0.48 ± 0.04 (44.6)	N.D.	0.22 ± 0.02 (4.1)	0.01 ± 0.00 (2.2)

Results are expressed as the mean ± 1 S.D. for a total of two to four determinations.

Values shown for succinate dehydrogenase, lactate dehydrogenase and arylsulfatase were obtained on newborn preparations and for NADPH-cytochrome$_c$ reductase, 5′-nucleotidase and UDP galactosyltransferase from adult. Value in parentheses represent the percentage of total activity recovered in each fraction. Fractions were prepared as described in the text.

[a]One unit, 1 nmol/min.
[b]N.D., not determined.
[c]Specific activity obtained for a purified plasma membrane preparation, 111.6 nmol/min/mg/protein (n=3).
[d]One unit, 1 pmol/min.

TABLE 2

Representative Phospholipid Compositions of Subcellular Fractions Obtained from Fetal Rabbit Lung

Fraction	Total phosolipid mg/g · dry lung	Percent of total lipid phosphorus							
		PC	LPC	SM	PS	PI	PG	PE	X
Tissue homogenate	77.8 ± 11.8	57.4 ± 0.6	0.4 ± 0.1	9.8 ± 1.1	5.1 ± 1.3	4.4 ± 0.1	0.6 ± 0.4	21.6 ± 0.8	0.9 ± 0.3
Nuclei, cell debris	48.0 ± 7.8	47.8 ± 0.7	1.2 ± 0.1	14.5 ± 0.8	3.9 ± 1.6	4.3 ± 0.2	0.8 ± 0.0	26.8 ± 1.1	1.0 ± 0.3
Mitochondrial-enriched	11.8 ± 1.8	53.6 ± 1.5	1.8 ± 0.9	9.2 ± 0.3	5.4 ± 1.6	4.8 ± 0.4	0.8 ± 0.4	23.3 ± 0.1	1.3 ± 0.6
Microsomal-enriched	2.7 ± 0.9	53.3 ± 4.1	0.5 ± 0.5	12.1 ± 1.5	6.7 ± 1.6	5.3 ± 0.8	1.2 ± 1.2	21.1 ± 3.5	N.D.[a]
Cytosol	1.3 ± 0.4	60.8 ± 1.3	0.1 ± 0.2	9.5 ± 0.9	5.5 ± 1.4	7.5 ± 0.4	1.3 ± 0.6	14.2 ± 1.3	1.1 ± 1.0
Lamellar body-enriched	10.9 ± 2.4	79.0 ± 0.5	0.1 ± 0.0	1.0 ± 0.1	2.8 ± 0.5	10.0 ± 0.8	1.2 ± 0.7	5.9 ± 0.1	0.2 ± 0.2
Alveolar lavage	3.0 ± 0.8	79.1 ± 1.4	N.D.	1.2 ± 0.7	1.5 ± 0.7	11.0 ± 0.9	1.4 ± 0.4	4.9 ± 0.8	0.2 ± 0.3

Fractions were prepared from fetal lung obtained at 31 days (term) gestation. Each value represents the mean ± 1 S.D. for a total of three to five determinations on each fraction.

Abbreviations: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; X, unidentified phospholipid.

[a]N.D., not detectable.

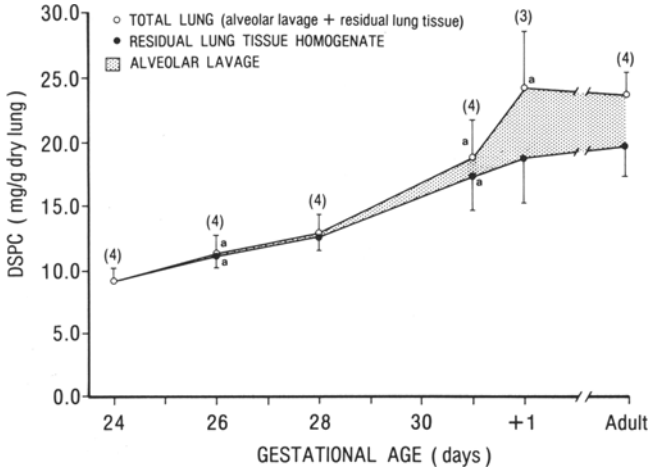

FIG. 1. DSPC content of alveolar lavage and unfractionated postlavaged lung tissue homogenate during perinatal development. Each value represents the mean ± 1 S.D. for the number of determinations shown in parentheses. (a) Significantly different from the value obtained at the previous gestational age ($p < 0.05$ by student's t-test).

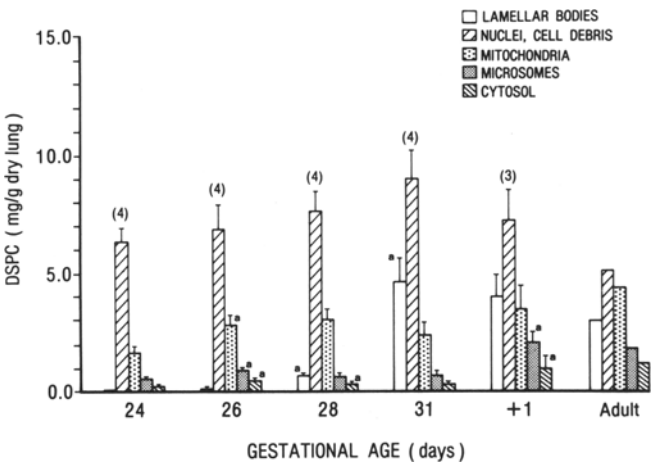

FIG. 2. DSPC content of subcellular fractions obtained from postlavaged lung tissue homogenate during perinatal development. Data representation and statistical analysis as in Figure 1.

The DSPC content of alveolar lavage and the unfractionated postlavaged lung tissue homogenate is illustrated in Figure 1. As shown, tissue homogenate DSPC progressively increased throughout fetal development, rising from 0.09 ± 0.91 to 17.45 ± 2.88 mg/g dry lung between 24 days gestation and term. Little change occurred following birth. Alveolar lavage DSPC also progressively increased during fetal development, rising from 0.02 ± 0.00 to 1.34 ± 0.36 mg/g dry lung between 26 days gestation and term. There was a further four-fold increase on the first postnatal day but no change thereafter. Total lung DSPC was calculated by summation of alveolar lavage and post-lavaged lung tissue homogenate values. The validity of this summation was established in a preliminary experiment in which the DSPC content was determined on unlavaged lung preparations. As indicated in the diagram (Fig. 1), the bulk of the total lung DSPC was recovered in the postlavaged lung tissue homogenate.

The DSPC content of the individual lung tissue fractions is shown in Figure 2. Significant developmental changes were observed for each fraction. On the 24th day of gestation, there was no isolable surfactant, and the bulk of the DSPC was recovered in the nuclear-cellular debris fraction. Between the 24th and 26th day of gestation, there was about a two-fold increase in mitochondrial, microsomal and cytosolic DSPC, with no change in the nuclear-cellular debris fraction. Surfactant was first isolable at 26 days gestation but contained less DSPC than the other subcellular fractions at this stage of development. From 26 days gestation to term, the DSPC content of the lamellar body fraction progressively and significantly increased, rising ca. six-fold (from 0.10 ± 0.02 to 0.67 ± 0.15 mg/g dry lung) between 26 and 28 days gestation and a further seven-fold (to 4.63 ± 1.06 mg/g dry lung) by term. There was little change in the DSPC content of the other subcellular fractions during this time. By the first postnatal day, both microsomal and cytosolic DSPC had again increased, but no significant change occurred in the other subcellular fractions.

TABLE 3

DSPC/Total PC Ratios of Rabbit Lung Subcellular Fractions During Perinatal Development

	DSPC/PC					
	24(4)[a]	26(4)	28(4)	31(4)	+1(3)	Adult (1)
Tissue homogenate	0.387 ± 0.030	0.401 ± 0.025	0.384 ± 0.028	0.436 ± 0.012	0.427 ± 0.026	0.427
Nuclei, cell debris	0.349 ± 0.012	0.383 ± 0.020	0.378 ± 0.027	0.399 ± 0.017	0.386 ± 0.031	0.333
Mitochondria	0.371 ± 0.014	0.390 ± 0.012	0.344 ± 0.010	0.384 ± 0.014	0.372 ± 0.008	0.347
Microsomes	0.504 ± 0.012	0.487 ± 0.029	0.385 ± 0.021	0.440 ± 0.014	0.492 ± 0.026	0.507
Cytosol	0.354 ± 0.018	0.390 ± 0.061	0.324 ± 0.011	0.357 ± 0.005	0.329 ± 0.034	0.310
Lamellar bodies	N.P.[b]	0.515 ± 0.072	0.579 ± 0.056	0.584 ± 0.015	0.505 ± 0.034	0.571
Alveolar lavage	N.D.[c]	0.459 ± 0.069	0.426 ± 0.048	0.570 ± 0.012	0.567 ± 0.018	0.572

Each value represents the mean ± 1 S.D. for the number of determinations shown.
Values underscored by a common line are significantly different ($p < 0.05$ by Student's t-test).

[a]Gestational age in days; number of determinations at each age in parentheses.
[b]N.P., none present.
[c]N.D., not determined.

Developmental changes were also found in the DSPC/total PC ratios of the individual subcellular fractions (Table 3). Each of the mitochondrial, microsomal and cytosolic ratios decreased on the 28th day of gestation and then increased. This trend was also observed in the unfractionated tissue homogenate. At each gestational age the largest DSPC/PC ratio was found in the lamellar body fraction. This ratio did not significantly change during fetal development but decreased (from 0.584 ± 0.015 to 0.505 ± 0.034) during the first postnatal day. The high value observed with the fetus was also observed in the adult. Alveolar lavage DSPC/PC ratios significantly increased between 28 days gestation and term, at which time the ratio was comparable to that obtained from the tissue-stored lamellar bodies.

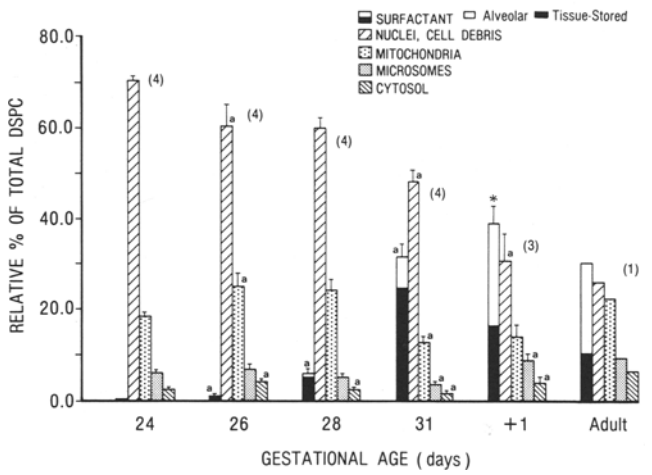

FIG. 3. Relative distribution of total lung DSPC over individual lung fractions during perinatal development. Total lung DSPC was calculated as the sum of alveolar lavage and postlavaged lung tissue DSPC. Data representation and statistical analysis as in Figure 1.

Using the data in Figures 1 and 2, the relative distribution of the total lung DSPC over the individual lung fractions was determined. Developmental changes were observed for each fraction (Fig. 3). While there was little change in the absolute content of DSPC in the nuclear-cellular debris fraction (Fig. 2), the relative proportion of total lung DSPC recovered in this fraction progressively decreased, falling from nearly 70% at 24 days gestation to about 30% by the first postnatal day. The relative proportion of total lung DSPC recovered in a surfactant pathway (tissue-stored plus alveolar) steadily increased so that by the first postnatal day this pathway contained the greatest proportion (almost 40%) of the total. At each gestational age, the mitochondrial-enriched fraction contained relatively more of the total DSPC than either the microsomes or cytosol. Developmental fluctuations were nevertheless observed in each of these subcellular fractions.

DISCUSSION

Compared to other tissues, the lung is particularly enriched in DSPC, a species of phosphatidylcholine in which both esterified fatty acids are saturated (29). Within the lung itself, this phospholipid represents the major constituent of the pulmonary surfactant, comprising up to 40–50% of the total phospholipids of this material (30). The surface tension-lowering properties of this unique material can largely be attributed to the presence of this phospholipid (1,5,6). Yet it has been shown that not all of the DSPC present in lung is associated with the surfactant complex (11–15). In the adult, for example, it has been shown that less than 50% of the total lung DSPC can be identified as being in a surfactant pathway (12–13).

In the developing fetus, surfactant production is initiated in the latter part of gestation and progressively increases toward term (2,3). The progressive accumulation of phospholipids (including DSPC) that has been observed during this time period (31–33) has generally been attributed to increased surfactant production. However, large quantities of DSPC appear to accumulate in lung tissue well before the normal onset of surfactant production (15,31–34), suggesting that, like the adult lung, the fetal lung contains considerable DSPC that is unrelated to the surfactant system.

In the fetal rabbit, surfactant synthesis is initiated on or about the 24th day of gestation (2,3), a fact that accounts for our inability to isolate a surfactant band at this gestational stage. In agreement with studies on both the rat (15,34) and rabbit (32), we did find large quantities of DSPC in the lung at this stage of development. While the bulk was recovered in the fraction designated as nuclear-cellular debris, significant levels were nevertheless recovered in the mitochondrial- and microsomal-enriched fractions as well as the cell cytosol.

At each gestational age, the largest proportion of the tissue homogenate DSPC was recovered in the nuclear-cellular debris fraction. While our present study indicates that this fraction contains not only the bulk of the cellular nuclei but other cellular constituents as well, previous studies in our laboratory have shown that very little surfactant becomes entrapped in this low-speed pellet fraction (16). In fact, it is for this reason that this very low centrifugal force has been adopted for our routine lamellar body isolation procedure. Furthermore, as large quantities of DSPC are recovered in the low-speed pellet fraction even before surfactant synthesis is initiated, it is reasonable to conclude that the bulk of the DSPC in this fraction is of nonsurfactant origin.

In agreement with others (15,31–34), we found that total tissue homogenate DSPC significantly increased during late fetal development. In addition, we have shown that not only the surfactant but the other subcellular fractions as well undergo dynamic changes in DSPC pool size during this critical stage of development. Prior to the onset of surfactant production, the overall increase in tissue homogenate DSPC appeared to be due to significant increases in the mitochondrial, microsomal and cytosolic pools. Once surfactant appears, then further increases in tissue homogenate DSPC can be attributed to increases in the surfactant pools. Following birth, on the other hand, the microsomal and cytosolic DSPC pools increase once more.

Individual surfactant constituents are synthesized in the endoplasmic reticulum of the alveolar Type II cell and transported via the Golgi apparatus for storage in the intracellular lamellar bodies, which are ultimately released to the alveolar space (4). The increased microsomal DSPC observed in the newborn could possibly represent newly synthesized material required for replenishment of the intracellular stores released to the airways following birth (2,3). Another mechanism by which depleted intracellular stores are replenished is via the uptake and reutilization of secreted surfactant constituents which are reprocessed by the Type II cell into new lamellar bodies (35). The increased cytosolic DSPC pool observed following birth could be due to this process. In considering either of these two possibilities, however, it is important to emphasize that, while the isolated surfactant originates from a single cell type, the other subcellular fractions no doubt originate from each of the more than 40 cell types present in lung (36). It is therefore difficult to determine whether the changes in DSPC pool size of these fractions are indeed a reflection of events occurring in the Type II cell or a more generalized effect. Studies with isolated Type II cells would be useful to clarify this issue.

It is interesting to note that while there was a dramatic release of DSPC to the alveoli following birth, there was not a concomitant decrease in the levels present in the intracellular surfactant stores. This suggests that the Type II cell may be still actively synthesizing and replenishing the intracellular stores. The decreased DSPC/PC ratio found in the intracellular stores also suggests that the newly synthesized material has not yet attained its full complement of the disaturated species. The newborns in the present study were killed less than 12 hr following birth. In a previous study (16) we found that it took at least 24 hr following birth to observe a depletion of intracellular stores, which were once more replenished in the next 24-hr period. While we did not determine DSPC levels in our previous study, results of our present study indicate that, at least in the adult, the high ratio observed in the late gestation fetus is once more attained. When this occurs in the postnatal period is not presently known.

Next to the tissue-stored surfactant, the mitochondrial-enriched fraction represents a major source of lung tissue DSPC. While this fraction is obviously contaminated by other subcellular organelles, we have previously shown that, like the nuclear-cellular debris fraction, negligible surfactant material is entrapped in this fraction (16). Also, considerable DSPC is recovered in this fraction prior to the onset of surfactant synthesis. As with the nuclear-cellular debris fraction, it is felt that the DSPC recovered in the mitochondrial-enriched fraction is not due to lamellar body contamination. Since this fraction is also enriched in Golgi, it could be argued that its high DSPC levels could be due to Golgi that are in the process of transferring surfactant constituents to the intracellular lamellar bodies. This may, in fact, be so for the late gestation fetus and early neonate but is unlikely to be the case in the 24-day gestation fetus.

DSPC is produced both by de novo synthesis and through remodeling of the monounsaturated phosphatidylcholine synthesized de novo (37-40). The latter mechanism occurs via two enzymatically catalyzed steps, the first involving a deacylation at the 2-position and the second involving a reacylation by an activated fatty acid (37-40). The first step is catalyzed by a phospholipase A_2, and the latter predominantly by a lysophosphatidylcholine acyltransferase. The former reaction appears to be the rate-limiting step. Phospholipase A_2 activity has been demonstrated in both purified lung microsomes and mitochondria (39), suggesting that both subcellular organelles are involved in the biosynthesis of DSPC. While it is reasonable to consider the translocation of microsomal DSPC to the intracellular lamellar bodies, this would not be likely for the mitochondrial pools. The possibility that the lamellar body itself is involved in its own DSPC production has been raised (38) but never fully established.

In conclusion, our results show that, in addition to the pulmonary surfactant, other lung subcellular fractions contain large DSPC pools that undergo dynamic changes in size during perinatal development. As DSPC is resistant to peroxidation, it may be that as well as performing its surface tension-lowering function as a major constituent of the pulmonary surfactant, it also represents a structural component for other subcellular organelles in a tissue such as lung that is exposed to high O_2 concentrations. While it is tempting to speculate that all subcellular fractions, including the lamellar bodies, are capable of their own DSPC production, further study would be necessary to clarify this point.

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REFERENCES

1. Goerke, J. (1974) *Biochim. Biophys. Acta 344*, 241-261.
2. Kikkawa, Y., Motoyama, E.K., and Gluck, L. (1968) *Am. J. Pathol. 52*, 177-192.
3. Kikkawa, Y., Kaibara, M., Motoyama, E.K., Orsalesi, M.M., and Cook, C.D. (1971) *Am. J. Pathol. 64*, 423-442.
4. Askin, F.B., and Kuhn, C. (1971) *Lab. Invest. 25*, 260-268.
5. Frosolono, M.F., Charms, B.L., Pawlowski, P., and Slivka, S. (1970) *J. Lipid Res. 11*, 439-457.
6. King, R.J., and Clements, J.A. (1972) *Am. J. Physiol. 223*, 715-726.
7. Torday, J.S., and Nielsen, H.C. (1981) *Biol. Neonate 39*, 266-271.
8. Today, J.S. (1985) *Biochim. Biophys. Acta 835*, 23-28.
9. Gilfillan, A.M., Harkes, A., and Hollingsworth, M. (1980) *J. Develop. Physiol. 2*, 101-110.
10. Landers, S., Corbet, A., Cregan, J., Frink, J., and Rudolph, A.J. (1984) *Am. Rev. Respir. Dis. 130*, 204-208.
11. Young, S.L., and Tierney, D.F. (1972) *Am. J. Physiol. 222*, 1539-1544.
12. King, R.J., Gikas, E.G., Ruch, J., and Clements, J.A. (1974) *Am. Rev. Respir. Dis. 110*, 273-281.
13. Young, S.L., Kremers, S.A., Apple, J.S., Crapo, J.D., and Brumley, G.W. (1981). *J. Appl. Physiol. 51*, 248-253.
14. Engle, M.J., Sanders, R.L., and Longmore, W.J. (1980) *Biochem. Biophys. Res. Commun. 94*, 23-28.

15. Faridy, E.E., Thliveris, J.A., and Morris, G.S. (1981) *Respir. Physiol. 45*, 55-66.
16. Oulton, M., Fraser, M., Dolphin, M., Yoon, R., and Faulkner, G. (1986) *J. Lipid Res. 27*, 602-612.
17. Oulton, M. (1979) *Am. J. Obstet. Gynecol. 135*, 337-343.
18. Oulton, M., Bent, A.E., Gray, J.H., Luther, E.R., and Peddle, L.J. (1982) *Am. J. Obstet. Gynecol. 142*, 684-691.
19. Mason, R.J., Nellenbogen, J., and Clements, J.A. (1976) *J. Lipid Res. 17*, 281-284.
20. Pennington, R.J. (1961) *Biochem. J. 80*, 649-654.
21. Omura, T., and Takesue, S. (1970) *J. Biochem. 67*, 249-257.
22. Avruch, J., and Wallach, D.F.H. (1971) *Biochim. Biophys. Acta 233*, 334-347.
23. Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M., and Berthet, J. (1974) *J. Cell. Biol. 61*, 188-200.
24. Jobe, A., Ikegami, M., Sarton-Miller, A., Jones, S., and Yu, G. (1981) *Biochim. Biophys. Acta 666*, 47-57.
25. Chang, P.L., Rosa, N.E., and Davidson, R.G. (1981) *Anal. Biochem. 117*, 382-389.
26. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265-275.
27. West, D.C., Sattar, A., and Kumar, S. (1985) *Anal. Biochem. 147*, 289-295.
28. Maeda, T., Balakrishnan, K., and Mehdi, S.W. (1983) *Biochim. Biophys. Acta 731*, 115-120.
29. Mason, R.J. (1973) *Am. Rev. Respir. Dis. 107*, 678-679.
30. Possmayer, F. (1984) in *Pulmonary Surfactant* (Robertson, B., Van Golde, L.M.G., and Batenburg, J.J., eds.) pp. 295-355, Elsevier, Amsterdam.
31. Soodsma, J.F., Mims, L.C., and Harlow, R.D. (1976) *Biochim. Biophys. Acta 424*, 159-167.
32. Hallman, M., and Ravio, K.J. (1975) *Biol. Neonate 27*, 329-338.
33. Funkhouser, J.D., and Hughes, E.R. (1978) *Biochim. Biophys. Acta 530*, 9-16.
34. Adamson, I.Y.R., and King, G.M. (1984) *Am. Rev. Respir. Dis. 129*, 130-134.
35. Jacobs, H., Jobe, A., Ikegami, M., and Conoway, D. (1983) J. *Biol. Chem. 258*, 4156-4165.
36. Weibel, E.R., Gehr, P., Haies, D., Gil, J., and Bachofen, M. (1976) in *Lung Cells and Disease* (Bouhuys, A., ed.) pp. 3-16, North Holland Publishing Co., Amsterdam/New York.
37. Tsao, F.H.C., and Zachman, R.D. (1977) *Pediatr. Res. 11*, 849-857.
38. Heath, M.F., and Jacobson, W. (1980) *Pediatr. Res. 14*, 849-857.
39. Garcia, A., Newkirk, J.D., and Mavis, R.D. (1975) *Biochem. Biophys. Res. Commun. 64*, 128-135.
40. Post, M., Schuurmans, E., Batenburg, J.J., and Van Golde, L.M.G. (1983) *Biochim. Biophys. Acta 750*, 68-77.

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COMMUNICATIONS

Differential Utilization of Long Chain Fatty Acids During Triacylglycerol Depletion. I. Rat Heart After Ischemic Perfusion[1]

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Rat hearts were perfused with Krebs-Henseleit buffer for 90 min according to the Langendorff procedure. Normoxic perfusion for 90 min resulted in minor changes in fatty acid composition and a decrease in residual heart triacylglycerol to 60% of preperfusion values. When the protocol included 30 min of slow perfusion-induced ischemia, the hearts were observed to be depleted of 89% of their initial triacylglycerol content. The triacylglycerol fatty acid composition (mg %) remained similar after compared to before perfusion except for a 121 mg % increase in stearic acid and a 225 mg % increase in arachidonic acid. The percentage composition of both fatty acids was significantly inversely correlated with the amount of triacylglycerol remaining in the heart after perfusion. Postperfusion, arachidonic acid and stearic acids were present at nearly 1:1 in the residual heart triacylglycerol, suggesting that a common mechanism may be involved in the selective retention of these fatty acids by the heart.
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Arachidonic acid (20:4n-6) in both liver and plasma triacylglycerol (TG) previously has been shown to be unique among other 16–20 carbon fatty acids in having a significant inverse correlation with the quantitative amount of TG in various species (1–3). Thus, when total liver TG is increased, e.g. after carbon tetrachloride injection or in essential fatty acid deficiency, the percentage composition of 20:4n-6 is lower but when total liver TG is significantly decreased, e.g. in starvation, 20:4n-6 is increased (4). While the percentage composition of 20:4n-6 varies inversely according to the total TG present, the quantitative amount of 20:4n-6 (μg/g) in TG is, except in extreme circumstances, very stable (2,3). Hence, the normal variability of the percentage composition of 20:4n-6 is attributable largely to the variability in the TG content of the major fatty acids in TG (palmitic-16:0, oleic-18:1n-9 and linoleic-18:2n-6). The marked decrease in total TG in the rat heart after perfusion in vitro with a glucose-free buffer (5) provided a different model in which the relation between 20:4n-6 and TG could be studied further.

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Abbreviations: 20:4n-6, arachidonic acid; GLC, gas liquid chromatography; 18:2n-6, linoleic acid; 18:1n-9, oleic acid; 16:0, palmitic acid; 16:1n-7, palmitoleic acid; 18:0, stearic acid; TG, triacylglycerol.

EXPERIMENTAL

Male Sprague-Dawley rats (200–250 g) were obtained from Charles River Canada (Montreal, Quebec) and were fed a semisynthetic diet of the following composition (g/kg); sucrose (555), casein (200), cellulose (100), corn oil (100), minerals (35) and vitamins (10) as recommended by the American Institute of Nutrition (6). After 10 weeks, the rats were decapitated and the hearts removed immediately and perfused retrograde in vitro using the Langendorff technique (7). The perfusion medium was oxygenated Krebs-Henseleit buffer containing glucose (7), which was pumped peristaltically through a cannula inserted into the base of the aorta and into the coronary arteries. Two perfusion protocols each were used for 90 min; normoxic (10 ml/min) or normoxic plus an ischemic episode (10 ml/min-30 min, followed by 1 ml/min-30 min, followed by 10 ml/min-30 min). For the first protocol (normoxic perfusion), six hearts were perfused, whereas in the second protocol (ischemic perfusion) 18 hearts were perfused. Fresh (unperfused) hearts and perfused hearts were homogenized in chloroform/methanol (2:1, v/v) containing 0.02% butylated hydroxytoluene as antioxidant. Total TG were separated from the total lipid extract by thin layer chromatography as previously described (8). Fatty acids were transmethylated with boron trifluoride-methanol and analyzed by GLC using a Hewlett-Packard 5880 under the column conditions previously described (8). TG were quantitated by GLC by the addition of an internal standard (triheptadecanoin) to the tissue homogenates prior to lipid separation.

RESULTS

Normoxic perfusion for 90 min led to 40% less residual TG ($p < 0.01$) and 50% higher 20:4n-6 ($p < 0.05$) in the heart compared to preperfusion values and 55% higher 20:4n-6 (Table 1). There was no significant correlation between the 20:4n-6 present in the heart TG before or after perfusion and the amount of TG present before or after perfusion.

The fatty acid composition (mg % and μg/g, wet weight) of rat heart TG before and after perfusion including 30 min ischemia is shown in Table 2. Proportionally, 16:0, 18:1n-9 and 18:2n-6 were not significantly different in the hearts before and after perfusion. Palmitoleic acid (16:1n-7) was significantly lower but stearic acid (18:0) and 20:4n-6 were significantly higher in the residual heart TG after perfusion (2.21 and 3.25 times, respectively, $p < 0.01$). N-3 fatty acids were not detected in heart TG before or after perfusion. Quantitatively, since the total TG was decreased by 89% in the hearts perfused with a 30-min ischemic episode, all the TG fatty acids were significantly lower after perfusion (Table 2). However, the quantitative fatty acid changes (μg/g) could be differentiated into two groups: A) 16:0, 16:1n-7, 18:1n-9 and 18:2n-6, which were

TABLE 1

Fatty Acid Composition of Rat Heart Total TG Before and After 90-min Normoxic Perfusion In Vitro

	Before[a]	After[b]	After/Before (%)
A. mg %			
18:0	2.7 ± 0.5[c]	3.1 ± 0.4	+15
18:1n-9	13.2 ± 1.9	13.6 ± 1.8	+6
18:2n-6	62.6 ± 5.1	60.4 ± 7.3	−4
20:4n-6	2.0 ± 0.5	3.1 ± 1.0*	+55
B. μg/g			
18:0	97 ± 47	63 ± 19	−35
18:1n-9	449 ± 289	293 ± 98	−35
18:2n-6	2168 ± 1411	1286 ± 974	−41
20:4n-6	73 ± 21	67 ± 17	−8
C. mg/g	3.48 ± 0.81	2.10 ± 0.43**	−40

Rat hearts were perfused for 90 min under normoxic conditions. Separate hearts were analyzed for fatty acid and lipid composition before (unperfused) and after perfusion (A, percentage composition of fatty acids; B, quantitative composition of fatty acids; C, total TG).

[a]n = 6.

[b]n = 6.

[c]mean ± SD.

*p < 0.05, **p < 0.01, Student's t-test.

TABLE 2

Fatty Acid Composition of Rat Heart Total TG Before and After Perfusion In Vitro

	Before[a]	After[b]	After/Before (%)
A. mg %			
16:0	12.8 ± 1.6[c]	13.8 ± 2.8	+8
16:1n-7	3.0 ± 0.9	1.8 ± 0.6**	−40
18:0	2.4 ± 0.5	5.3 ± 2.4**	+121
18:1n-9	14.9 ± 2.4	13.6 ± 1.5	−9
18:2n-6	61.8 ± 4.7	59.2 ± 8.5	−4
20:4n-6	2.0 ± 0.6	6.5 ± 3.6**	+225
B. μg/g			
16:0	563 ± 295	62 ± 22**	−89
16:1n-7	147 ± 101	8 ± 5**	−95
18:0	100 ± 43	20 ± 5**	−80
18:1n-9	648 ± 353	60 ± 31**	−91
18:2n-6	2757 ± 1542	284 ± 132**	−90
20:4n-6	76 ± 27	23 ± 8**	−70
C. mg/g	4.13 ± 2.40	0.46 ± 0.22**	−89

Rat hearts were perfused for 90 min, including a 30-min ischemic episode. Separate hearts were analyzed for lipids and fatty acids before (unperfused) and after perfusion (A, percentage composition—mg %; B, quantitative composition—μg/g, wet weight; C, total TG—mg/g).

[a]n = 12.

[b]n = 18.

[c]mean ± SD.

**p < 0.01, Student's t-test.

decreased 89–95%; B) 18:0 and 20:4n-6, which were decreased 80% and 70%, respectively. Hence, compared to the net decrease of 89% in the TG fatty acids as a whole, 18:0 and 20:4n-6 selectively were retained in the residual TG remaining in the heart after ischemic perfusion.

A significant inverse correlation was observed between the mg % 20:4n-6 in TG and total heart TG (mg/g) in both fresh (unperfused) hearts and in hearts after perfusion using the ischemic episode. This correlation was also evident for 18:0 (Fig. 1). Whereas 100 μg/g 18:0 and 76 μg/g 20:4n-6 (ratio—1.32) were present in the heart TG before perfusion, after perfusion 20 μg/g 18:0 and 23 μg/g 20:4n-6 (ratio—0.89) were present. In unperfused hearts, there was no correlation between 18:0 and 20:4n-6 in heart TG ($r = -0.03$) but after perfusion (including 30 min ischemia), the correlation coefficient was $r = +0.70$ ($p < 0.01$, Fig. 2). Thus, the residual TG in the heart contained almost equal amounts of 18:0 and 20:4n-6 after ischemic perfusion, and in individual hearts (postperfusion), the two fatty acids were highly correlated. Comparison of the

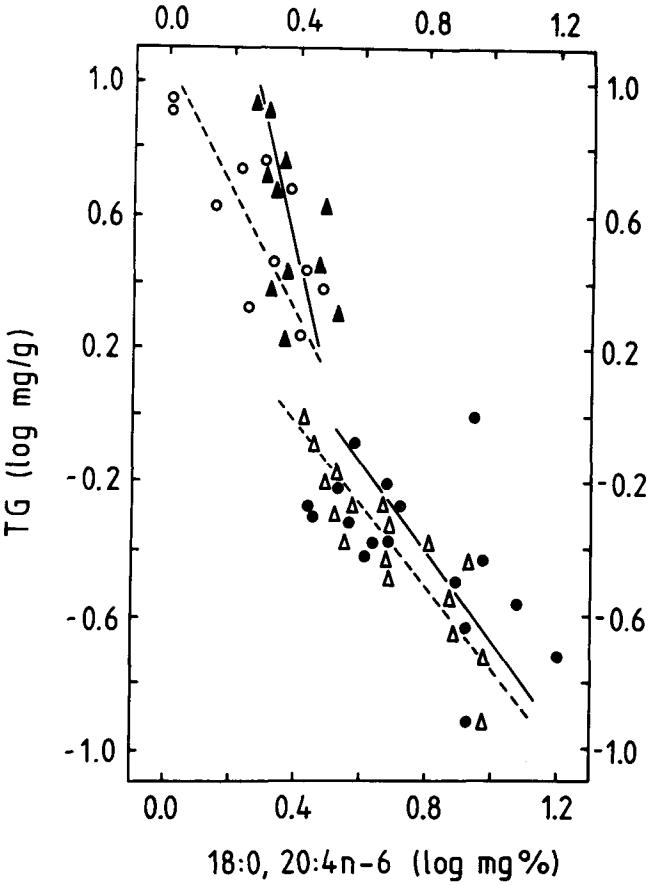

FIG. 1. Linear regression analysis of the relation between the proportional composition of arachidonic acid (20:4n-6, log mg %) and stearic acid (18:0, log mg %) of TG and total TG in rat hearts before (unperfused) and after perfusion in vitro (including 30 min ischemia). Correlation coefficients, regression equations and number of samples, before perfusion: (20:4n-6—○— — —, $r = -0.75$; $y = -0.48X + 0.55$, $p < 0.001$; $n = 11$; 18:0—▲——, $r = -0.58$, $y = -0.20X + 0.49$, $p < 0.01$, $n = 11$); after perfusion: (20:4n-6—●——; $r = 0.57$; $y = -0.56X + 0.53$; $p < 0.001$; $n = 17$; 18:0—△— — —; $r = -0.89$; $y = -0.72X + 0.40$; $p < 0.001$; $n = 17$).

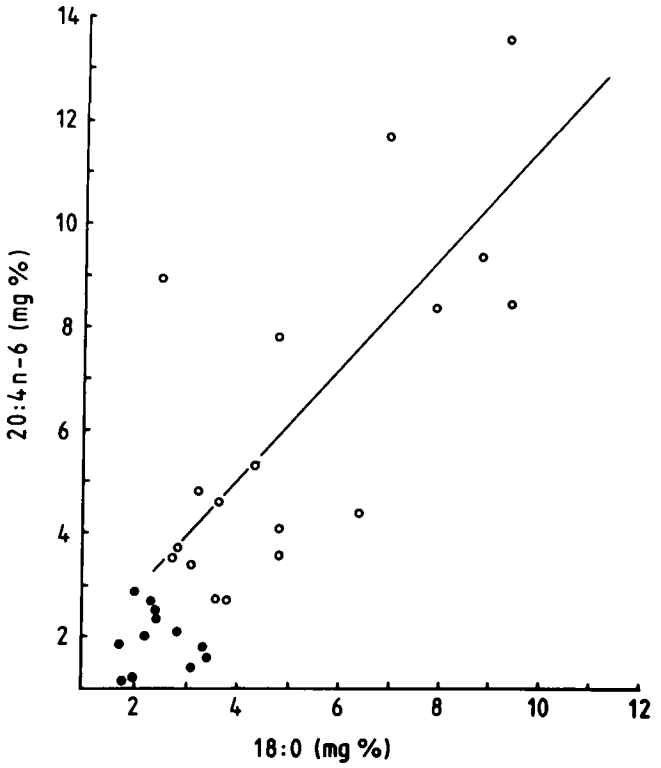

FIG. 2. Linear regression analysis of the relation between the proportional composition of arachidonic acid (20:4n-6) and stearic acid (18:0) in rat heart TG (●) before (unperfused, n = 12) and (○) after (n = 18) in vitro perfusion of the hearts (including 30 min ischemia). Correlation coefficients and regression equations: Before—($r = -0.03$, not significant). After—($r = +0.70$, $y = 0.55X + 0.27$, $p < 0.001$).

TG levels and composition of 20:4n-6 and 18:0 after perfusion in hearts perfused with or without the ischemic episode showed that the mg % of 20:4n-6 and 18:0 was significantly higher ($p < 0.01$) and the TG (mg/g) was significantly lower in hearts perfused with the ischemic episode compared to those perfused without ischemia.

DISCUSSION

These results confirm the previously reported significant inverse correlation between the mg % of 20:4n-6 in TG and total TG in plasma and liver (1-3) and extend the relationship to include the rat heart perfused in vitro. Because of the marked TG depletion, which occurs within 90 min of perfusion (only in the protocol that included 30 min of ischemic perfusion), this model appears ideal to investigate the utilization of long chain fatty acids from TG species in the heart. That ischemia is responsible for the markedly lower residual heart TG levels after 90-min perfusion is suggested by the significantly higher residual TG levels and the lack of significant changes in the fatty acid composition (except 20:4n-6) of heart TG after normoxic perfusion (Table 1). Glucose in the perfusion medium was the only exogenous energy source for the heart. By providing only 10% of the medium/min compared to normoxic perfusion, ischemic perfusion presumably required the heart to utilize endogenous energy sources, e.g. TG, more than during normoxic perfusion, hence depleting heart TG significantly more than in normoxic perfusion (Tables 1 and 2).

The retention of 20% of the original 18:0 and 30% of the original 20:4n-6 such that nearly equal amounts remained in the residual heart TG after ischemic perfusion suggests that at least two pools of 20:4n-6 may exist in rat heart TG, one of which is more readily hydrolyzed along with fatty acids such as 16:0, 18:1n-9 and 18:2n-6 during in vitro perfusion. The other 20:4n-6 pool appears to be retained more avidly possibly in combination with 18:0. That the retention of 20:4n-6 and 18:0 in rat heart TG after perfusion, including a 30 min ischemic episode, may be interrelated is suggested by the similar amount of these two fatty acids remaining in the residual TG and the significant positive correlation between these two fatty acids in the heart TG after perfusion (Fig. 2).

TG previously have been shown to be an important endogenous energy source for heart metabolism (9). Long-chain fatty acids within the total TG pool previously have not been distinguished according to their availability for hydrolysis for energy utilization by the heart. However, previous data have indicated that part of the total TG in heart is unavailable for energy metabolism (5,10). The present data support this view and suggest that, especially in the rat heart perfused in vitro using a protocol that includes a 30-min ischemic episode, a proportion of 20:4n-6 and 18:0 (20-30%) may be unavailable for hydrolysis. This may be because they are hydrolyzed differentially by TG lipases in comparison with those containing 16:0, 18:1n-9, 18:2n-6 or 16:1n-7. Alternatively, some areas of heart tissue may not be accessible for TG hydrolysis, thus causing a shift in fatty acid composition after perfusion with a medium not supplying exogenous fatty acids. Future research will address this issue.

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REFERENCES

1. Cunnane, S.C., Huang, Y-S., and Manku, M.S. (1986) *Biochim. Biophys. Acta 876*, 183-186.
2. Cunnane, S.C. (1986) *Prog. Lipid Res. 25*, 91-92.
3. Cunnane, S.C., McAdoo, K.R., and Prohaska, J.R. (1986) *J. Nutr. 116*, 1248-1254.
4. Takeuchi, H., Ito, A., and Muramatsu, K. (1985) *Agric. Biol. Chem. 49*, 1853-1855.
5. Olsen, R.E., and Hoeschen, R.J. (1967) *Biochem. J. 103*, 796-801.
6. American Institute of Nutrition (1977) *J. Nutr. 107*, 1340-1348.
7. Karmazyn, M., and Dhalla, N.S. (1983) *Can. J. Physiol. Pharmacol. 61*, 1207-1225.
8. Cunnane, S.C., Manku, M.S., and Horrobin, D.F. (1984) *J. Nutr. 114*, 1754-1761.
9. Neely, J.R., and Morgan, H.E. (1974) *Ann. Rev. Physiol. 36*, 413-459.
10. Neely, J.R., Rovetto, M.J., and Oram, J.F. (1972) *Prog. Cardiovasc. Dis. 15*, 289-329.

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Fluorescence Formation from Hydroperoxide of Phosphatidylcholine with Amino Compound

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The hydroperoxides of methyl linoleate, 1-palmitoyl-2-linoleoyl-phosphatidylcholine and trilinolein each produced similar fluorescent substances through reaction with amino compounds after decomposition by heme methyl ester. Fluorescent substances formed from methyl linoleate with 1-aminopentane revealed characteristic fluorescence peaks on HPLC, while those obtained from 1-palmitoyl-2-linoleoyl-phosphatidylcholine and trilinolein were not eluted under the same conditions. However, when both of these fluorescent substances were transesterified to methyl ester, the same fluorescence peaks were observed. This result suggests that fluorescent substances formed from oxidized membrane lipids with amino compounds remain attached to phospholipids without being released from their glycerol backbone.
***Lipids* 23, 65–67 (1988).**

Polyunsaturated fatty acids (PUFA) initially are oxidized to produce hydroperoxides (HPO) by both enzymic and nonenzymic oxidations and then the HPO produced are degraded further into secondary oxidation products (SP), including many functional aldehydes or malondialdehyde, during the oxidation process (1,2). These lipid peroxides and/or their secondary decomposition products are known to react with various amino compounds, bringing about many forms of biological damage (3). Fluorescent lipofuscin pigments associated with aging in animal tissues also have been assumed to be derived from the reaction of lipid peroxides with amino compounds (4). Fujimoto et al. recently reported that the degradation of HPO prepared from methyl linoleate (ML) in the presence of metals, hemin or ascorbic acid is involved closely in fluorescence formation through reaction with DNA (5). They also suggested that the important structural feature associated with the fluorescence formation appears to be conjugated diene HPO and not unconjugated diene isomers produced during photosensitized oxidation. Therefore, HPO derived from PUFA substituted at the 2-glycerol position of phospholipids in biological membranes (6,7) may play an important role in the formation of fluorescent substances under physiological conditions.

We recently have demonstrated that SP prepared from autoxidized methyl linolenate and ML are involved in the formation of fluorescent substances through reaction with amino compounds (8,9). The high-resolution mass spectrum of the fluorescent substance (FS-II) formed from the SP with 1-aminopentane (1-AP) as a model system showed the empirical formula to be $C_{24}H_{41}NO_4$ with a molecular weight of 407 and the presence of a methyl ester group originating from ML in the structure of the fluorescent substance (Iio, unpublished data). These results suggest that the precursor available for the fluorescence formation may be a 19-carbon length compound having a methyl ester group originating from ML without breakdown into low molecular weight compounds. Fukuzawa et al. also recently have reported that a new type of fluorescent substance was produced during oxidation in the presence of amino compounds through reaction of 12-keto-oleic acid produced from oleic acid without breakdown into low molecular weight compounds (10). In the present study, we demonstrated the formation of fluorescent substances through reaction of HPO prepared from ML, 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC) and trilinolein (TL) with 1-AP after decomposition by heme methyl ester (HM) as a model system.

MATERIALS AND METHODS

Materials. Linoleic acid, PLPC, TL, hemin (type I) and lipoxygenase (type I) were purchased from Sigma Chemical Co. (St. Louis, MO). 1-AP and 5% methanolic *m*-trifluoromethylpheny-trimethyl ammonium hydroxide were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). High performance liquid chromatography (HPLC) grade solvents from Kanto Kagaku Co., Ltd. (Tokyo) were used for HPLC separations throughout all experiments. HM was prepared by the method of Ortiz de Montellano et al. (11).

Preparation of HPO from ML, PLPC and TL. Two mg of PLPC or TL suspended in 1 ml of 100 mM deoxycholate solution was incubated with 9 ml of 0.1 M borate buffer (pH 9.0) containing 2 mg of lipoxygenase at 30 C for 30 min (12). The products were extracted with chloroform/methanol (2:1), and each HPO was purified by HPLC or thin layer chromatography (TLC). The purification of HPO from oxidized PLPC was carried out on a μ-Bondapak FAA (3.9 × 300 mm, Waters Associates, Milford, MA), using methanol/water (9:1) at a flow rate of 1 ml/min (13). HPO of TL was subjected to TLC (kieselgel 60, Merck, Darmstadt, FRG) developed with petroleum ether/ethyl ether/28% ammonia (55:45:2) (14). 13-Hydroperoxylinoleic acid was prepared from linoleic acid using soybean lipoxygenase in 0.1 M borate buffer (pH 9.0) (15). The products were methylated with diazomethane and purified by TLC using hexane/ethyl ether/acetic acid (60:40:1) as a solvent system. SP of ML were prepared from autoxidized ML by silica gel column chromatography according to the method of Terao and Matsushita (16). Each purified HPO was estimated using molar absorbance values of 24,500 at 233 nm (17).

Formation of fluorescent substances. Each HPO (100 nmol) prepared from ML, PLPC or TL was preincubated with or without HM (1 nmol) in 2 ml of methanol at 37 C for 45 min, and then 1 ml of 1-AP (1 μmol) in methanol solution was added to the preincubation mixtures. SP also were incubated with 1-AP in 3 ml of methanol. Fluorescence developed at 37 C during 20 hr. Fluorescence spectra were measured with a Hitachi

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Abbreviations: HM, heme methyl ester; HPLC, high performance liquid chromatography; HPO, hydroperoxides; ML, methyl linoleate; PLPC, 1-palmitoyl-2-linoleoyl-phosphatidylcholine; PUFA, polyunsaturated fatty acids; SP, secondary oxidation products; TL, trilinolein; TLC, thin layer chromatography.

MPF-3 fluorescence spectrophotometer, and the intensity was expressed as a percentage of that of a quinine sulfate standard (0.1 μg/ml in 0.1 N H_2SO_4). HPLC was performed on a μ-Bondasphere phenyl column (3.9 × 150 mm, Waters) using acetonitrile/water (50:50) as the mobile phase at a flow rate of 0.7 ml/min. Fluorescence peaks were monitored with a Hitachi 650-10LC fluorescence spectrophotometer with excitation and emission maxima at 350 nm and 420 nm, respectively. All of the fluorescent substances also were subjected to TLC and developed with chloroform/methanol (9:1).

Transesterification of fluorescent material. To 0.1 ml of fluorescent material, 0.85 ml of methanol and 0.05 ml of 5% methanolic *m*-trifluoromethylphenyltrimethyl ammonium hydroxide were added, and this mixture was allowed to stand at 20 C (18). Transesterification usually was completed within 45 min. The mixture was evaporated under a nitrogen stream and redissolved in 1 ml of chloroform. This chloroform solution was applied to a Sep-pak Si cartridge (Waters), and the cartridge was washed out with 8 ml of chloroform to remove the transesterification reagent. The fluorescent substance was eluted with 4 ml of chloroform/methanol (9:1) and subjected to reversed-phase HPLC under the same conditions.

RESULTS AND DISCUSSION

When purified HPO prepared from ML, PLPC and TL were incubated separately with 1-AP in methanol solution at 37 C for 20 hr after decomposition by HM, marked formation of fluorescent substances was observed. These fluorescent substances showed the same fluorescence spectra with excitation and emission maxima at 340–350 nm and 410–420 nm, respectively, consistent with that observed for SP prepared from autoxidized ML (Fig. 1). None of the HPO preincubated without HM produced any fluorescent substances. This result suggests that HPO from the unsaturated esterified fatty acids in PLPC and TL are related equally to the formation of fluorescent substances through reaction with amino compounds in the presence of HM.

However, HPLC analysis of each fluorescent product showed independent elution profiles, as shown in Figure 2A. The fluorescent substances formed from HPO of ML with 1-AP after decomposition by HM revealed two major fluorescence peaks at retention times of 5.0 min (FS-I) and 10.5 min (FS-II), respectively. On the other

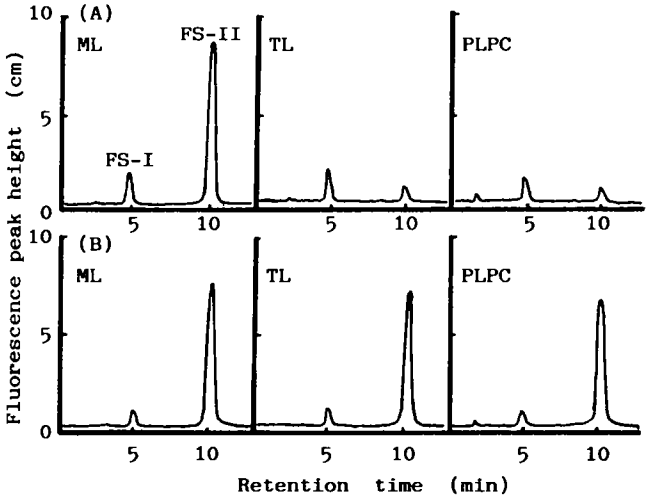

FIG. 2. HPLC of fluorescent substances formed from HPO of ML, PLPC and TL with 1-AP. HPLC was performed on a μ-Bondasphere phenyl column, and elution was carried out with acetonitrile/water (50:50) at a flow rate of 0.7 ml/min. Aliquots of the fluorescent substances were subjected to HPLC before (A) and after (B) transesterification using the procedures described in Materials and Methods.

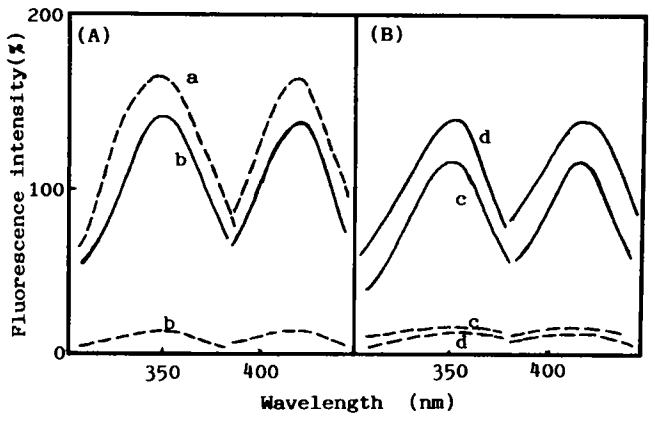

FIG. 1. Fluorescence spectra of fluorescent substances formed from SP or HPO of ML, PLPC and TL with 1-AP. HPO of ML (A-b), PLPC (B-c) and TL (B-d) were incubated with 1-AP at 37 C for 20 hr after preincubation with (——) or without (···) HM. SP prepared from autoxidized ML by silica gel column chromatography also was incubated with 1-AP at 37 C for 20 hr (A-a).

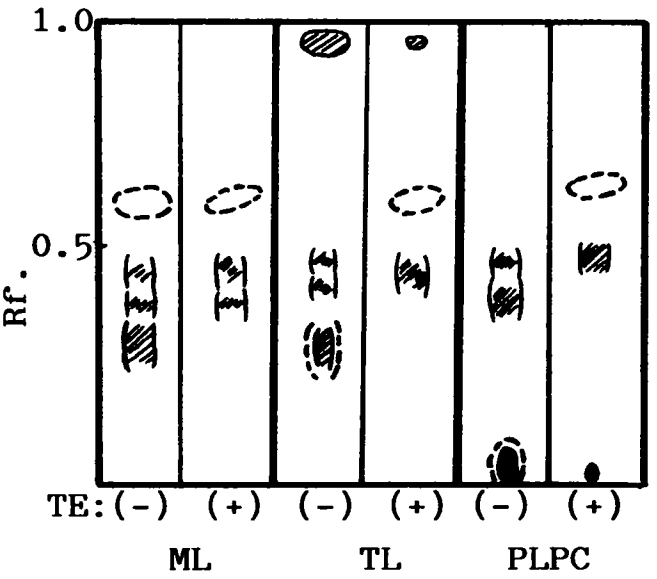

FIG. 3. TLC of flurescent substances produced from HPO of ML, PLPC and TL with 1-AP. Aliquots of fluorescent substances were subjected to TLC before (−) and after (+) transesterification (TE) and developed with chloroform/methanol (9:1). Fluorescent spots (broken circle) were detected by excitation at around 360 nm, while other spots (hatched circle) were detected using iodine vapor.

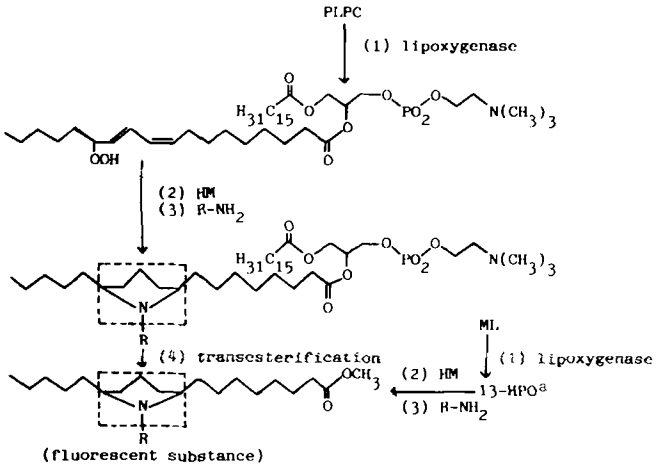

FIG. 4. Proposed mechanism for formation of fluorescent substances formed from PLPC with amino compound. The procedures (1-4) for fluorescence formation are described in Materials and Methods. The chromophobic structure enclosed with a dotted line is at present obscure. 13-HPO[a], 13-hydroperoxy methyl linoleate.

hand, the fluorescent substances derived from HPO of PLPC and TL with 1-AP showed only small fluorescence peaks at these retention times, even though samples with the same fluorescence intensities as that of ML were used for HPLC analysis and the major fluorescence peak was eluted with methanol (data not shown).

Therefore, these fluorescent substances produced from HPO of ML, PLPC and TL were transesterified to methyl esters in methanol solution and subjected to HPLC under the same conditions as those described above. Both fluorescent substances produced from HPO of PLPC and TL also were eluted at similar retention times of 10.5 min as that of FS-II obtained from HPO of ML (Fig. 2B). However, a negligible amount of FS-I was detected on HPLC after transesterification because FS-I was unstable.

TLC analysis of both the fluorescent substances produced from the HPO of PLPC and TL after transesterification to methyl ester revealed one major fluorescence spot (Rf 0.60) corresponding to FS-II formed from HPO of ML with 1-AP, while the two native fluorescent substances present before transesterification showed fluorescence spots distinct from that of FS-II on TLC (Fig. 3). The appearance of this fluorescent substance (FS-II) from the two fluorescent substances after transesterification strongly supports the possibility that HPO produced from unsaturated esterified fatty acids in phospholipids or triglycerides are involved in the fluorescence formation without being released from their glycerol backbone.

It is well known that phosphatidylcholine and phosphatidylethanolamine are the most common types of membrane lipids and that linoleic acid and arachidonic acid are abundant PUFA constituents of these phospholipids (19,20). Therefore, HPO produced from these PUFA in phospholipids by lipoxygenase or autoxidation (6,7) seem to be a primary cause of accumulation of fluorescent substances in biomembranes under physiological conditions. Nielsen reported that peroxidized phospholipid (cardiolipin) produced the fluorescent substance through reaction with albumin (21). Moreover, Shimasaki et al. have reported that the fluorescent chromolipids formed during lipid peroxidation of liposomes by ferrous ions and ascorbic acid remain in liposomal membranes (22). These reports, in addition to the results here, suggest that the hydrolytic release of unsaturated esterified fatty acids that usually are substituted at the 2-glycerol position in phospholipids is not essential for the fluorescence formation occurring in biomembranes. We recently demonstrated that both 9-hydroperoxy ML and 13-hydroperoxy ML, which are the major conjugated diene HPO produced during autoxidation of ML, formed the same fluorescent substances through reaction with 1-AP after preincubation with HM (23).

Based on the above results and the structural information mentioned above, we propose a possible mechanism for the fluorescence formation occurring during oxidation of unsaturated phospholipids in biomembranes using 13-hydroperoxides of PLPC and ML as a model compound, as shown in Figure 4. It seems likely that the precursor available for fluorescence formation keeps the original 18-carbon length of linoleic acid substituted onto phospholipids, but the structure of this precursor has not been established in detail yet.

Although fluorescent chromolipids accumulating with age in animal tissues are suggested to be produced in biomembranes, our results may support the view that fluorescent substances produced from oxidized membrane phospholipids are localized in biomembranes and accumulated without release from biomembranes.

REFERENCES

1. Gunstone, F.D. (1984) *J. Am. Oil Chem. Soc. 61,* 441-447.
2. Frankel, E.N. (1984) *J. Am. Oil Chem. Soc. 61,* 1908-1917.
3. Tappel, A.L. (1973) *Fed. Proc. 32,* 1870-1874.
4. Kikugawa, K. (1986) *Adv. Free Rad. Biol. Med. 2,* 389-417.
5. Fujimoto, K., Neff, W.E., and Frankel, E.N. (1984) *Biochim. Biophys. Acta 795,* 100-107.
6. Porter, N.A. (1984) in *Methods in Enzymology* (Packer, L., and Board, A., eds.) Vol. 105, pp. 273-282, Academic Press, New York, NY.
7. Porter, N.A., and Wagner, C.R. (1986) *Adv. Free Rad. Biol. Med. 2,* 283-323.
8. Yoden, K., Iio, T., and Tabata, T. (1981) *Yakugaku Zasshi 101,* 437-442.
9. Iio, T., and Yoden, K. (1987) *Life Sci. 40,* 2297-2302.
10. Fukuzawa, K., Kishikawa, K., Tokumura, A., Tsukatani, H., and Shibuya, M. (1985) *Lipids 20,* 854-861.
11. Ortiz de Montellano, R.P., and Mico, B.A. (1980) *Mol. Pharmacol. 18,* 128-135.
12. Eskola, J., and Laakso, S. (1983) *Biochim. Biophys. Acta 751,* 305-311.
13. Crawford, C.G., Plattner, R.D., Sessa, D.J., and Rackis, J.J. (1980) *Lipids 15,* 91-94.
14. Shode, J., Izutani, S., and Matsushita, S. (1973) *Agr. Biol. Chem. 37,* 1979-1981.
15. Funk, M.O., Isasc, R., and Porter, N.A. (1976) *Lipids 11,* 113-116.
16. Terao, J., and Matsushita, S. (1977) *J. Am. Oil Chem. Soc. 54,* 234-238.
17. Johnston, A.E., Zilch, K.T., Selke, E., and Dutton, H.J. (1961) *J. Am. Oil Chem. Soc. 38,* 367-371.
18. McCreary, D.K., Kossa, W.C., Ramachandran, S., and Kurtz, R.R. (1978) *J. Chromatogr. Sci. 16,* 329-331.
19. Belina, H., Cooper, S.D., Farkas, R., and Feuer, G. (1975) *Biochem. Pharmacol. 24,* 301-303.
20. Waskell, L., Koblin, D., and Canova-Davis, E. (1982) *Lipids 17,* 317-320.
21. Nielsen, H. (1978) *Lipids 13,* 253-258.
22. Shimasaki, H., Ueta, N., Mowri, H., and Inoue, K. (1984) *Biochim. Biophys. Acta 792,* 123-129.
23. Iio, T., Yoden, K., and Tabata, T. (1987) *Chem. Pharm. Bull. 35,* 5015-5910.

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Regulation of Squalene Epoxidase Activity by Membrane Fatty Acid Composition in Yeast

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Depriving *Saccharomyces cerevisiae* strain GL7 of exogenous unsaturated fatty acid supplements causes this sterol biosynthetic mutant to accumulate squalene at the expense of squalene epoxide and squalene diepoxide. To further characterize the apparent relationship between squalene epoxidase activity and membrane fatty acid composition, a variety of unsaturated fatty acids differing in their chain lengths and in the positions and orientation (*cis* or *trans*) of their double bonds were tested for their ability to promote turnover of endogenous squalene in cells previously deprived of olefinic supplements. All of the unsaturated fatty acids tested were found to restore squalene epoxidase activity but there were marked differences in their efficacies that best were correlated with the extent to which they reduced the medium chain (C-10 + C-12) saturated fatty acid content of cellular phospholipids. Additional studies demonstrated that de novo protein synthesis was required for the restoration of squalene epoxidase activity in unsaturated fatty acid-deprived cells.
***Lipids* 23, 68-71 (1988).**

Sterols such as cholesterol and ergosterol are ubiquitous components of animal and fungal membranes, respectively; they are thought to play an important role in modulating bilayer fluidity (1). In animal cells, the regulation of cholesterol biosynthesis is complex and resides primarily at the level of 3-hydroxy-3-methylglutaryl-coenzyme A reductase [HMG-CoA reductase] (2), although other enzymes also may serve as regulatory elements (3). Modulation of HMG-CoA reductase activity seems to occur via a variety of molecular mechanisms, including changes in enzyme synthesis, enzyme degradation and phosphorylation-dephosphorylation (4). A direct regulation of enzyme activity via changes in membrane fluidity also has been proposed (5) but the data regarding such a mechanism are conflicting (6). In contrast to the situation in animal cells, much less is known regarding the regulation of sterol synthesis in yeast and fungi.

Due to two different mutations, *Saccharomyces cerevisiae* strain GL7 is unable to synthesize either ergosterol or unsaturated fatty acids and requires exogenous lipid supplements for long-term growth (7). Nevertheless, the mutant can grow for extended periods of time in the absence of unsaturated fatty acids (8); strain GL7 appears to compensate for the lack of olefinic fatty acid supplements by replacing most of its membrane unsaturated fatty acids with medium chain (C-10 and C-12) saturated fatty acids (8). Under such conditions, strain GL7 grows more slowly, demonstrates a marked "clumpiness" and synthesizes squalene rather than the squalene epoxide [2,3-oxidosqualene] and squalene diepoxide [2,3;22,23-dioxidosqualene] lipids that normally accumulate due to the mutant's deficiency in 2,3-oxidosqualene-lanosterol cyclase activity (7,9). Conversely, short-term cholesterol deprivation favored the synthesis of squalene diepoxide (9). These data suggested that sterol synthesis in yeast also may be regulated by changes in membrane fluidity.

To further characterize the relationship between membrane lipid composition and squalene epoxidase activity in yeast, we now have compared various olefinic fatty acids for their ability to restore squalene epoxidase activity in strain GL7 cells, which previously had been deprived of unsaturated fatty acid supplements. Our results demonstrate that an inverse relationship exists between squalene epoxidase activity and the proportion of phospholipids containing C-10 + C-12 fatty acids in yeast cell membranes.

MATERIALS AND METHODS

Culture conditions. Saccharomyces cerevisiae strain GL7 (MAT a *gal2/erg*12-1/*heme*3-6 [7]) was grown at 30 C with shaking in a defined medium supplemented with methionine and detergent-solubilized cholesterol and unsaturated fatty acid supplements (9). The pulse-chase protocol used to compare the ability of various unsaturated fatty acids to restore squalene epoxidase activity was described previously (9). Briefly, log phase cells grown in the presence of cholesterol (10 μg/ml) and oleic acid (18:1Δ^9; 20 μg/ml) were washed twice with media and resuspended to the desired density in media containing cholesterol but lacking unsaturated fatty acid supplements. The cells were incubated under these deprivation conditions for 12 hr, after which 50 ml aliquots were pulsed for one to three hr with 10 μCi of [1-^{14}C] acetate (55 mCi/mmol; New England Nuclear, Boston, MA). The pulse was terminated with 1 M sodium acetate, and the cells were collected by centrifugation and washed twice prior to being resuspended to the same cell density in cholesterol-supplemented media either lacking unsaturated fatty acid supplements or containing one of a variety of unsaturated fatty acids (final concentration 20 μg/ml). At this point (designated as time 0), >90% of the [^{14}C] acetate incorporated into nonsaponifiable lipids was associated with squalene (9).

In vivo assay of squalene epoxidase activity. Yeast cells containing endogenously synthesized ^{14}C-labeled squalene were incubated in the absence or presence of unsaturated fatty acid supplements, and either at two-hr intervals or after a total incubation time of six hr cells were harvested and their cellular lipids were isolated and analyzed by thin layer chromatography (TLC; see below) to determine the amount of ^{14}C-labeled squalene that had been converted to squalene epoxide and squalene diepoxide. In most cases, the level of squalene epoxidase activity in vivo is reported as the percent of ^{14}C-labeled squalene present at time 0 that was converted to squalene epoxide + squalene diepoxide during the chase.

Lipid analyses. Total cellular lipids were extracted from lyophilized yeast cells and separated by TLC as described

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previously (9). ^{14}C-Labeled lipids were localized by either autoradiography using Kodak XAR-5 film, or by the use of a linear radioactivity detector (Bioscan, Washington, DC), and quantitated by either liquid scintillation counting or by use of the Bioscan instrument.

Following separation of cellular lipids by TLC, phospholipids remaining at the origin were treated with BF_3-MeOH at 100 C for five min, and the fatty acyl methyl esters were extracted into pentane. Fatty acyl methyl esters were analyzed by gas liquid chromatography (GLC) using a Perkin-Elmer Sigma 3B gas chromatograph equipped with a six-ft glass column packed with SP2330 (Supelco) and a program having an initial time of 0 min, a ramp rate of 5 C/min, a final time of 11 min, initial temperature of 135 C and a final temperature of 175 C.

RESULTS AND DISCUSSION

Using the pulse-chase protocol described previously (9), a variety of unsaturated fatty acids were compared for their ability to restore squalene epoxidase activity in vivo. As shown in Figure 1, all of the unsaturated fatty acids tested promoted the conversion of squalene to squalene epoxide and squalene diepoxide but there were marked differences in their efficacies. Cetoleic acid (20:1) only was effective marginally in restoring squalene epoxidase activity, while linoleic acid (18:2) was the most effective of the unsaturated fatty acids tested. The other unsaturated fatty acids tested (myristoleic acid, 14:1; palmitoleic acid, 16:1; oleic acid, 18:1Δ^9; petroselenic acid, 18:1Δ^6; and elaidic acid, *trans*-18:1Δ^9) were found to be intermediate in their effectiveness. Thus, while squalene epoxidase appears to require unsaturated fatty acids for activity, the requirement is nonspecific in that it can be satisfied by unsaturated fatty acids differing in their chain lengths as well as in the position, number and orientation of their unsaturations.

One possibility for the differing efficacies of the unsaturated fatty acids is that during the six-hr chase period, they were incorporated to varying extents reflecting differences in their suitabilities as substrates for fatty acid transport or phospholipid biosynthesis. To examine this possibility, the phospholipid fatty acid profiles of the cells shown in Figure 1 and the cells of three additional experiments were determined by GLC. When the unsaturated fatty acid compositions of cellular phospholipids were plotted as a function of the amount of ^{14}C-labeled squalene converted to squalene epoxide and squalene diepoxide during the six-hr chase period (Fig. 2A), a positive correlation (r = 0.743) was observed consistent with unsaturated fatty acids being required for squalene epoxidase activity. From the best-fit line shown in Figure 2A, values for the amounts of phospholipid unsaturated fatty acids that would support minimal and maximal squalene epoxidase activity were determined to be ca. 20% and 70%, respectively. Conversely, if the medium-chain (C-10 + C-12) saturated fatty acid content of the same cells was plotted as a function of squalene

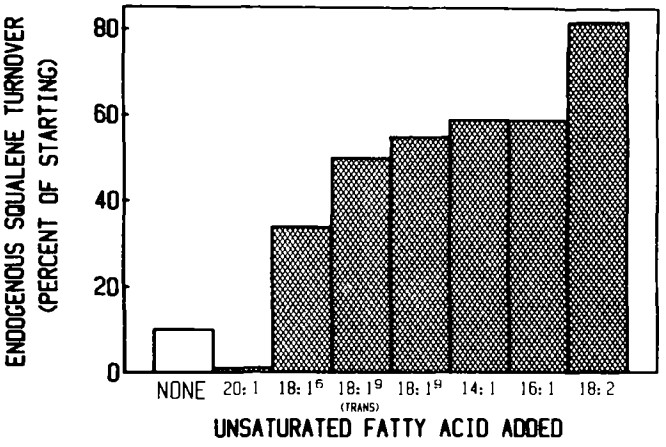

FIG. 1. Effect of unsaturated fatty acid supplements on squalene epoxidase activity. *S. cerevisae* strain GL7 was incubated for 14 hr in the absence of exogenous unsaturated fatty acids, with [1-^{14}C] acetate being added during the final two hr (pulse period) to label endogenous squalene. The cells subsequently were washed and incubated in the presence of one of the indicated unsaturated fatty acid supplements. After an additional six-hr incubation (chase period), cellular lipids were extracted and analyzed by TLC. The data are presented as the percent of endogenous ^{14}C-labeled squalene converted to squalene epoxide + squalene diepoxide.

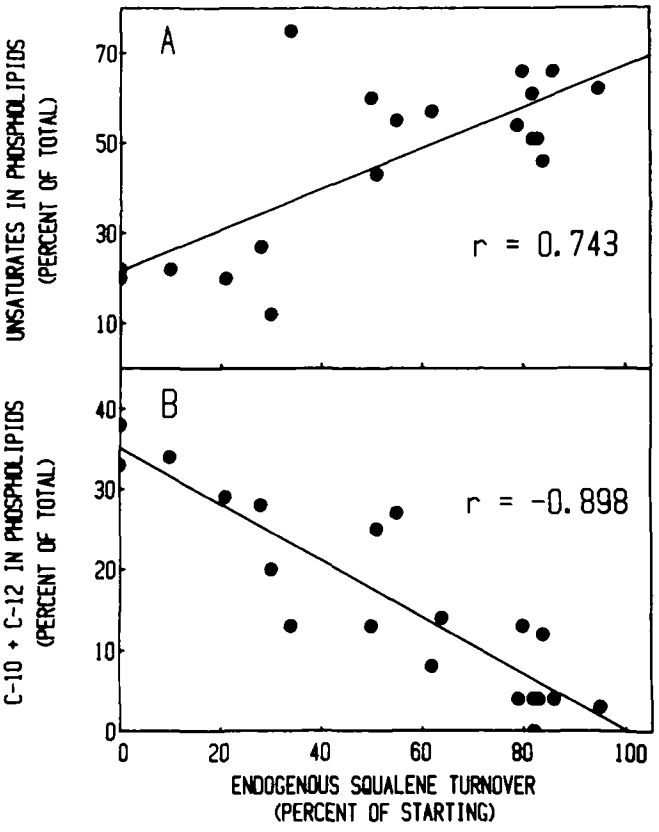

FIG. 2. Correlation between membrane fatty acid composition and squalene epoxidase activity. Phospholipid fatty acid compositions were determined for the cells analyzed in Figure 1, as well as for cells from three other identical experiments. The amount of endogenous ^{14}C-labeled squalene converted to squalene epoxide + squalene diepoxide during the six-hr chase period was plotted on the X-axis vs either the percent of unsaturated fatty acids (Panel A) or the percent of medium chain saturated fatty acids (C-10 + C-12) present in total cellular phospholipids on the Y-axis (Panel B). Linear regression analyses were performed to determine best-fit lines.

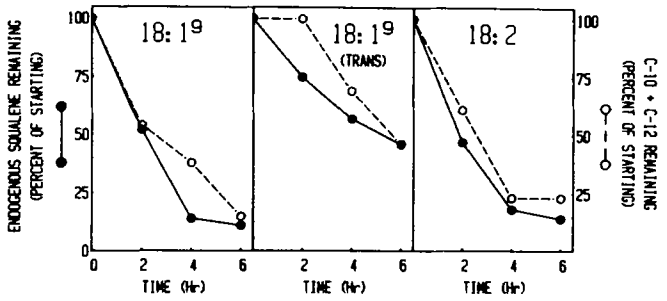

FIG. 3. Time-course of squalene and C-10 + C-12 fatty acid turnover in unsaturated fatty acid-supplemented cells. *S. cerevisiae* strain GL7 was subjected to the same pulse-chase protocol as described in the legend to Figure 1. During the chase period, cells were incubated in the presence of either oleic acid (18:1⁹), elaidic acid (*trans*-18:1⁹) or linoleic acid (18:2) and at two-hr intervals aliquots of the cultures were analyzed for turnover of both ^{14}C-labeled squalene and C-10 + C-12 fatty acids. The data are presented as the percent of either endogenous ^{14}C-labeled squalene or C-10 + C-12 fatty acids remaining at the indicated times.

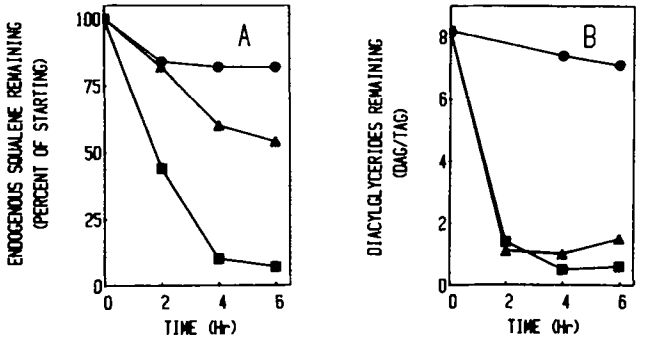

FIG. 4. Effect of cycloheximide on the turnover of squalene and diacylglycerides. *S. cerevisiae* strain GL7 was subjected to the same pulse-chase protocol as described in the legend to Figure 1. During the chase period, cells were incubated either in the absence (●) or presence (■) of oleic acid, or in the presence of oleic acid and cycloheximide (▲) and at 2-hr intervals aliquots of the cultures were analyzed for turnover of both ^{14}C-labeled squalene and ^{14}C-labeled diacylglycerides. The data are presented as either the percent of ^{14}C-labeled squalene remaining (left panel) or as the ratio of ^{14}C-labeled diacylglycerides to ^{14}C-labeled triacylglycerides (right panel) at the indicated times.

epoxidase activity (Fig. 2B), a negative correlation ($r = -0.898$) was obtained. The best-fit line shown in Figure 2B indicates that squalene epoxidase should be active maximally when C-10 + C-12 are absent from yeast membranes but it should be totally inactive when the amount of C-10 + C-12 fatty acids approaches 35% of total phospholipid fatty acids. Since unsaturated fatty acids and C-10 + C-12 fatty acids are incorporated primarily into the 2-position of cellular phospholipids (10; S.L. Brint and T.M. Buttke, unpublished data), it appears that squalene epoxidase activity totally is inhibited when the ratio of mixed-chain saturated phospholipids (i.e. those containing palmitic acid at the 1-position and a C-10 or C-12 fatty acid at the 2-position [11]) to phospholipids containing unsaturated fatty acids approaches 2:1.

To further correlate squalene epoxidase activity with the levels of C-10 + C-12 fatty acids in cellular phospholipids, strain GL7 was subjected to the same pulse-chase protocol described above, except that during the chase period aliquots were removed at two-hr intervals for subsequent lipid analyses. The data in Figure 3 show that in cells supplemented with 18:1Δ^9, *trans*-18:1Δ^9 or 18:2, squalene turnover parallels the disappearance of C-10 and C-12 fatty acids from membrane phospholipids, further demonstrating the relationship between membrane fatty acid composition and squalene epoxidase activity in yeast.

The restoration of squalene epoxidase activity that occurred following the readdition of olefinic supplements to unsaturated fatty acid-deprived cells could result from either the activation of pre-existing, but previously inactive squalene epoxidase enzyme, or from the de novo synthesis of new enzyme. In an attempt to distinguish between these two possibilities, strain GL7 was subjected to the usual pulse-chase protocol except that during the chase period cycloheximide was added to block de novo protein synthesis. At two hr intervals, aliquots were removed from the cultures, and the effects of the protein synthesis inhibitor on squalene turnover were determined. In the experiment shown in Figure 4A, the addition of cycloheximide (5 μg/ml) to 18:1Δ^9-supplemented cells inhibited the conversion of squalene to squalene epoxide and squalene diepoxide by 63% relative to control cells. This experiment was performed three times with cycloheximide inhibiting squalene epoxidase activity by an average of 71%. That cycloheximide was not merely blocking unsaturated fatty acid uptake or affecting cellular lipid metabolism in general was confirmed by assessing the effects of the drug on diacylglyceride turnover. As described previously (9), during unsaturated fatty acid deprivation, strain GL7 accumulated significant levels of diacylglycerides at the expense of triacylglycerides. Following the readdition of unsaturated fatty acid supplements, the accumulated diacylglycerides were rapidly turned over with the labeled diacylglycerides being chased primarily into triacylglycerides (9). As shown in Figure 4B, the addition of 5 μg/ml of cycloheximide had no effect on diacylglyceride turnover (for three different experiments the average inhibition was <5%), indicating that the drug did not inhibit lipid metabolism nonspecifically. These data suggest that at least a portion of the squalene epoxidase activity recovered following the addition of unsaturated fatty acids is derived from de novo protein synthesis.

In summary, the inhibition of squalene epoxidase activity that accompanies unsaturated fatty acid deprivation in *S. cerevisiae* strain GL7 seems to result from a replacement of olefinic residues at position 2 of membrane phospholipids with medium chain (C-10 + C-12) saturated fatty acids. The failure of the mixed-chain saturated phospholipids to support squalene epoxidase activity in yeast may stem from their tendency to adopt a unique bilayer structure (11).

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REFERENCES

1. Bloch, K. (1983) *CRC Crit. Rev. Biochem. 14,* 47-92.
2. Rudney, H., and Sexton, R.C. (1986) *Ann. Rev. Nutr. 6,* 245-272.
3. Faust, J.R., Goldstein, J.L., and Brown, M.S. (1979) *Proc. Natl. Acad. Sci. USA 76,* 5018-5022.
4. Kennelly, P.J., and Rodwell, V.W. (1985) *J. Lipid Res. 26,* 903-914.
5. Davis, P.J., and Poznansky, M.J. (1987) *Proc. Natl. Acad. Sci. USA 84,* 118-121.
6. Hashimoto, S., Drevon, C.A., Einstein, D.B., Bennett, J.S., Dayton, S., and Steinberg, D. (1983) *Biochim. Biophys. Acta 754,* 126-133.
7. Gollub, E.G., Lin, K., Doyan, J., Aldersberg, M., and Sprinson, D. (1977) *J. Biol. Chem. 252,* 2846-2854.
8. Buttke, T.M., Reynolds, R., and Pyle, A.L. (1982) *Lipids 17,* 361-366.
9. Buttke, T.M., and Pyle, A.L. (1982) *J. Bacteriol. 152,* 747-756.
10. Meyer, F., and Bloch, K. (1963) *J. Biol. Chem. 238,* 2654-2659.
11. Small, D.M. (1984) *J. Lipid Res. 25,* 1490-1500.

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Effects of Dietary Peanut Oil on Serum Lipoprotein Patterns of Rats

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Oils prepared from two varieties of peanuts and from a hybrid corn having linoleic acid concentrations substantially different from the respective commercial oils were compared with commercial oils for their effects on serum lipids of weanling female rats. In the first experiment, serum lipid patterns appeared to reflect linoleic acid content of the dietary oil. However, with a longer feeding period in the second experiment, serum lipid patterns were determined by the plant source of the dietary oil rather than its linoleic acid content; all peanut oils differed from both corn oils in their physiological effects. Diets containing triglyceride, hydrocarbon and sterol fractions obtained by liquid chromatography of peanut and corn oils were fed to female rats. The data provide no evidence that the hydrocarbon or sterol fractions of peanut oil are responsible for its unusual atherogenicity when fed as the sole fat source or that similar fractions from corn oil are protective against the effects of peanut oil.
Lipids 23, 72-75 (1988).

Atherogenicity of edible fats has been related to the degree of saturation of their component fatty acids (1); more unsaturated oils are reputed to be less cholesterolemic than those with fewer double bonds. However, peanut oil has been reported as unexpectedly atherogenic for rats, rhesus monkeys and rabbits (22), and its physiological effect is likened to that of a saturated fat. Corn oil was reported by Kritchevsky (3) to be the least atherogenic of several oils tested. In particular, peanut oil was more atherogenic than corn oil to rabbits (4) although both commercial oils contain about 80% unsaturated (oleic plus linoleic) fatty acids.

The hypocholesterolemic effects of unsaturated fats have been attributed to their content of polyunsaturated fatty acids (5). Linoleic acid is the major polyunsaturated fatty acid of corn and peanut oil, comprising about 60% of the totally fatty acids in commercial corn oil. Worthington and Hammons (6) reported values of 14 to 40% linoleic acid for oil derived from several peanut varieties.

Kritchevsky et al. (7) suggested that the triglyceride structure of peanut oil might be responsible for its physiological effects and presented preliminary evidence that randomized peanut oil was no more atherogenic than corn oil.

Although the acylglycerols of most seed oils make up at least 95% of the oils by weight, the remaining fraction consists of a mixture of classes of compounds including steroid and nonsteroid substances (8). The atherogenicity of peanut oil could be attributable to an alkali labile or soluble nonacylglycerol fraction that is lost during refining or randomization.

The major objective of these studies was to try to identify the component of peanut oil that is responsible for its atherogenicity. In the first two experiments, effects of concentration of dietary linoleic acid were evaluated by comparing oils from two genotypes of peanut in which the content of this polyunsaturated fatty acid was near the lower and upper limits reported for this species. Subsequent to the first experiment, grain from a hybrid corn with an unusually low linoleic acid concentration in its oil became available. Since the linoleic acid content of oil from the hybrid corn was about equal to that of oil from the high-linoleic acid peanut genotype, a comparison of their physiological effects seemed particularly appropriate.

Experiment 3 was designed to test nontriglyceride components of peanut oil for their effects on rat serum lipoprotein patterns.

A corollary to the hypothesis of an active atherogenic factor in peanut oil is the possibility that corn oil contains a factor that suppresses atherogenesis. Therefore, in the fourth study corn oil was fractionated to determine if some nontriglyceride part of this oil might modify the physiological response of the test animals to dietary peanut oil.

MATERIALS AND METHODS

Distribution of fatty acids in oils was determined by gas liquid chromatography following methylation in a mixture of methanol, benzene and sulfuric acid (9). Analyses were performed on a Microtek 220 chromatograph equipped with dual flame ionization detectors and a 180 × 0.4 cm glass column packed with 10% Silar 7CP on 80/100 Chromosorb W(AW)(DMCS) maintained at 210 C.

Native peanut and commercial corn oils containing about 32% and 59% linoleic acid, respectively, were fractionated using liquid chromatography on silica gel columns as described by Worthington and Hitchcock (8). The fraction, containing hydrocarbons and sterol esters (HC), was eluted with 2% ethyl acetate in hexane, the triacylglycerol (TG) fraction with 10% ethyl acetate in hexane, and the fraction containing free sterols (FS) with ethyl acetate. The percentages of these fractions in peanut and corn oils were HC, 0.5% and 0.8%; TG, 97.0% and 95.6%; and FS, 2.5% and 3.6%, respectively.

The experimental diets (Table 1) were adequate nutritionally for the rat and differed only in source of oil, which made up 20% of the weight of the diets. In experiments 1 and 2, the oil for each diet was derived solely from one of the selected peanut or corn sources. The components of the dietary oils for experiments 3 and 4 are set forth in Table 2 along with the designation for each diet that will be used henceforth. Whole oils and TG fractions were used as the sole source of lipid. HC and FS fractions were added in proportion to their content in the whole oil with the remainder of the dietary lipid provided by whole oil from the other oil seed (Table 2). During the feeding periods, an accurate measure of food intake by each rat was obtained.

Weanling female rats (Crl:CDR [SD]BR, Charles River Breeding Laboratories, Wilmington, MA) were housed

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Abbreviations: FS, free sterols; HC, hydrocarbon and sterol esters; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triacylglycerol.

TABLE 1

Composition of Diets

Ingredient	g/kg
Casein	200
Methionine	3
Vitamin mix[a]	22
Mineral mix[b]	35
Cellulose[a]	100
Sucrose, powdered	170
Corn starch	250
Cholesterol	20
Oil	200

[a]Vitamin Diet Fortification Mixture, U.S. Biochemical Corp. (Cleveland, OH).
[b]Mix UCB-1Rb, ref. 13.

TABLE 2

Composition of Lipids in Diets Fed in Experiments 3 and 4

		Lipid composition			
		Peanut oil		Corn oil	
#	Designation	Fraction	g/kg	Fraction	g/kg
1	Peanut	Whole	200	None	
2	Peanut TG	TG	200	None	
3	Peanut HC	HC	1	Whole	200
4	Peanut FS	FS	5	Whole	195
5	Corn	None		Whole	200
6	Corn TG	None		TG	200
7	Corn HC	Whole	198	HC	1.6
8	Corn FS	Whole	193	FS	7.2

Abbreviations: TG, triglyceride fraction; HC, hydrocarbon and sterol ester fraction; FS, free sterol fraction.

individually in stainless steel cages with wire mesh floors and provided with deionized water and diet ad libitum. The environment was maintained at 21 ± 1 C with 12-hr light-dark cycles. Following two days of acclimatization after being received in the laboratory, 10 rats were allotted on the basis of body weight to each dietary treatment group.

At the end of the test period, the rats were fasted overnight (15 to 18 hr) and 100–200 μl blood samples, obtained by amputating the tip of the tail, were collected in Microtainers (Becton-Dickerson, Rutherford, NJ) and centrifuged to separate serum for assessment of serum lipoprotein patterns. The rats again were fed for several days and then fasted overnight. With the animals under pentobarbital anesthesia, blood was obtained by a heart puncture in heparinized syringes for serum cholesterol determinations.

Lipoprotein patterns were evaluated by elecrophoresis on Aragose films (Agarose Universal Electrophoresis Film, Corning Medical, Medfield, MA). Two 1 μl aliquots of serum were added sequentially to sample wells, allowing sufficient time for diffusion of the first before addition of the second. Each sample was analyzed in triplicate

TABLE 3

Growth Performance of Rats Fed Diets Containing Corn and Peanut Oils

Diet treatment	Weight gain (g)	Feed intake (g)	Feed efficiency (gain/intake)
Experiment 1, 31 days			
Commercial corn oil	147	414	0.35
White skin peanut oil	141	418	0.34
Jenkins jumbo peanut oil	153	425	0.36
(Pooled SE[b])	(5.5)	(9.3)	(0.007)
Experiment 2, 49 days			
Commercial corn oil	193	578	0.33
Hybrid corn oil	195	588	0.33
White skin peanut oil	180	553	0.32
Commercial peanut oil	183	574	0.32
Jenkins jumbo peanut oil	177	536	0.33
(Pooled SE)	(5.8)	(13.94)	(0.006)
Experiment 3, 46 days			
Peanut	188	685bc	0.27a
Peanut TG	199	863a	0.23b
Peanut HC	179	635c	0.28a
Peanut FS	183	675bc	0.26a
Corn	210	740b	0.28a
(Pooled SE)	(9.3)	(22.7)	(0.006)
Experiment 4, 45 days			
Peanut	178	644	0.28
Peanut TG	174	673	0.26
Peanut HC	168	647	0.26
Peanut FS	164	621	0.26
Corn	161	685	0.24
Corn TG	179	653	0.27
Corn HC	167	633	0.26
Corn FS	173	640	0.27
(Pooled SE)	(7.4)	(19.9)	(0.006)

[a]Ten rats per treatment. Values for each parameter within an experiment followed by no common letter are significantly different at $P \leq 0.05$.
[b]Pooled standard error of dietary treatment means.

with each replication being applied to a different film. Electrophoretograms were developed and scanned as described previously (10). The low density lipoprotein (LDL) and high density lipoprotein (HDL) peak heights were measured in mm, and LDL/HDL ratios were calculated and averaged across replicates. Though the peak area may be a more accurate measure of the amount of a substance on a chromatogram than is peak height, the peaks measured were symmetrical, and their heights were considered adequately representative of lipoprotein quantity for the comparisons made in these studies.

Total cholesterol was determined in whole serum by a modification of the Abell-Kendall procedure (11). LDL were precipitated with heparin and manganese (12) and HDL cholesterol in the supernatant serum determined as described (11).

RESULTS AND DISCUSSION

The experimental diets promoted rapid growth of the young female rats throughout the test periods (Table 3).

TABLE 4

Lipoproteins and Cholesterol in Serum of Rats Fed Diets Containing Corn and Peanut Oils[a]

Diet treatment		Blood serum components			
			Cholesterol (mg/ml)		
Oil	18:2[b] (%)	Lipoprotein LDL/HDL	Total	HDL	HDL/total, %
		Experiment 1, 31 days			
Commercial corn	60.9	2.1b	0.4b	0.10a	28.2a
White skin peanut	38.7	3.7ab	0.6ab	0.08b	19.8a
Jenkins jumbo peanut	20.8	5.5a	0.8a	0.04c	6.2b
(Pooled SE[c])		(0.90)	(0.09)	(0.015)	(3.6)
		Experiment 2, 49 days			
Commercial corn	58.8	7.8b	0.9	0.14a	14.1a
Hybrid corn	40.4	6.6b	1.1	0.13a	11.3a
White skin peanut	38.7	10.2a	1.0	0.06b	6.2b
Commercial peanut	33.1	12.0a	1.4	0.08b	5.9b
Jenkins jumbo peanut	23.4	11.5a	1.3	0.07b	5.9b
(Pooled SE)		(1.45)	(0.18)	(0.02)	(2.1)
		Experiment 3, 46 days			
Peanut		35.1a	1.6a	0.05b	
Peanut TG		15.8b	1.8a	0.06b	
Peanut HC		4.5b	0.7b	0.10a	
Peanut FS		8.0b	1.2ab	0.06b	
Corn		8.5b	0.8b	0.10a	
(Pooled SE)		(5.1)	(0.25)	(0.008)	
		Experiment 4, 45 days			
Peanut		26.2a	2.2		
Peanut TG		18.3a	1.6		
Peanut HC		7.7b	1.6		
Peanut FS		6.5b	1.3		
Corn		7.3b	1.4		
Corn TG		7.1b	1.0		
Corn HC		22.5a	1.8		
Corn FS		22.9a	2.0		
(Pooled SE)		(3.7)	(0.3)		

[a]Ten rats per treatment. Values for each parameter within an experiment followed by no common letter are significantly different at $P \leq 0.05$.

[b]Linoleic acid content (% of total fatty acids) of oil in diet.

[c]Pooled standard error of dietary treatment means.

There were no statistically significant differences in weight gain due to dietary treatments in any of the experiments. Feed intake varied somewhat among the diet groups in experiment 3, resulting in a lower rate of conversion of feed to body mass by the group of rats fed peanut TG as a source of dietary fat.

The Jenkins jumbo peanut oil with 21% linoleic acid evoked significantly different serum lipoprotein and cholesterol responses in the female rats than did the commercial corn oil with 61% linoleic acid in experiment 1 (Table 4). The high LDL/HDL ratio and low ratio of HDL/total cholesterol in serum of rats fed this peanut oil are compatible with current theories of blood lipid composition that is conducive to atherogenesis. Serum lipid data for rats fed the White skin peanut oil in this experiment were intermediate between those for rats fed the other two oils. Since the linoleic acid content of the former oil also was intermediate between that of the latter two oils, these data could be interpreted to support the concept of an inverse relationship between polyunsaturated (linoleic) fatty acid content of an oil and its atherogenicity.

The feeding period was extended from 31 days in experiment 1 to 49 days in experiment 2. The higher values for LDL/HDL ratios found in the latter over those in the former reflect the longer exposure of the rats to the diets.

In experiment 2, the LDL/HDL ratio was higher for rats fed each of the diets containing peanut oil than for rats fed either of the diets containing corn oil. Despite the nearly identical linoleic acid content of the hybrid corn oil and the White skin peanut, rats fed diets containing the two oils exhibited distinctively different lipoprotein ratios and serum HDL cholesterol contents.

Although the commercial corn oil contained 59% linoleic acid and the hybrid corn oil 40%, both oils elicited

similar LDL/HDL ratios and serum cholesterol concentrations in the rats. In like manner, the three peanut oils with linoleic acid concentrations of 39%, 33% and 23% produced similar values for the serum lipid parameters among the groups of rats fed diets containing each of them.

The lack of significant difference in the first experiment between response of rats fed the White skin peanut oil with 39% linoleic acid and those fed corn oil could be attributable to the shorter time in which the rats were subjected to the dietary treatments. The serum lipid values characteristic of peanut oil may require more than 31 days for full development under conditions of these experiments. Also, the onset of differential response to type of dietary oil may occur several weeks after weaning in the rat.

In these studies, the physiological response to source of dietary oil appears to be characteristic of the species of plant from which the oil was derived rather than to the fatty acid composition of oil from individual genotypes.

The female rats in experiments 3 and 4 remained healthy and presented no overt evidence that the components of peanut and corn oil derived from the fractionation procedure were detrimental to growth performance of the animals. The LDL/HDL ratios were highest for the groups of rats fed whole peanut oil in both experiments (Table 4). Addition of the hydrocarbon fraction or FS fraction of peanut oil to diets containing whole corn oil resulted in lipoprotein patterns similar to those of rats fed only corn oil. Thus, the data from these studies provide no evidence that the hydrocarbon and FS fractions of peanut oil contain substances that can elevate the serum LDL/HDL ratio of rats.

In the last study, rats fed diets having the hydrocarbon fraction or the FS fraction of corn oil added to diets containing peanut oil had serum lipoprotein patterns similar to those of rats fed only peanut oil. These fractions of corn oil provided the rats with no protection against the physiological effects of peanut oil on their serum lipid profiles.

The effects of peanut TG fraction on the LDL/HDL ratio is ambiguous in these studies. The mean value for the ratio was between that for whole peanut and whole corn oil in both studies but was not discrete statistically from both of the oils in either. The results obtained could indicate partial loss of one agent or loss of one or more of multiple agents responsible for the metabolic effects of peanut oil during its fractionation by the described procedure. Further evidence relating to this hypothesis could be obtained in a future experiment in which the three components of fractionated peanut oil were recombined and effects on LDL/HDL ratio of the mixture compared with the original oil.

Total serum cholesterol values tended to be higher in rats fed diets containing 19-20% peanut oil or peanut TG than in those fed diets with most of the fat from corn, though those fed diets containing corn oil supplemented with peanut FS in experiment 3 and with peanut hydrocarbons in experiment 4 evidence somewhat higher sterol values than expected.

The data presented indicate that the physiological reponse of young female rats to dietary peanut oil is different from that to corn as a source of dietary lipids. The serum lipoprotein patterns and cholesterol levels elicited by peanut oil currently are believed to be conducive to atherogenesis and, thus, to be undesirable. The data do not substantiate the hypothesis that linoleic acid content of dietary fat is a decisive factor in determining its atherogenecity.

The fractionation scheme applied to the oils separated from the major TG component one portion of substances less polar than TG (e.g., HC) and another portion of components that would be slightly more polar than the TG such as FS. The data presented provide no evidence that either of these latter minor components of peanut oil are responsible for its atherogenecity or that either of the similar portions of corn oil counteract the physiological effects of peanut oil. The data do indicate that the elevation in serum LDL/HDL ratio might be somewhat less with the purified peanut TG fraction than with whole peanut oil. A different fractionation scheme might be found to reduce further the adverse effect of dietary peanut oil.

REFERENCES

1. McGill, H.C., and Matt, G.E. (1976) in *Present Knowledge of Nutrition*, 4th ed., The Nutrition Foundation, Washington, DC.
2. Anonymous (1983) *Nutr. Rev. 41,* 322-323.
3. Kritchevsky, D. (1970) *Am. J. Clin. Nutr. 23,* 1105-1110.
4. Kritchevsky, D., Tepper, S.A., Kim, H.K., Story, J.A., Vesselinovitch, D., and Wessler, R.W. (1976) *Exp. Mol. Pathol. 24,* 375-391.
5. Kris-Etherton, P.M., Ho, C.Y., and Fosmire, M.A. (1984) *J. Nutr. 114,* 1675-1682.
6. Worthington, R.E., and Hammons, R.O. (1977) *J. Am. Oil Chem. Soc. 54,* 105A-108A.
7. Anonymous (1983) *Nutr. Rev. 41,* 322-323.
8. Worthington, R.E., and Hitchcock, H.L. (1984) *J. Am. Oil Chem. Soc. 61,* 1085-1088.
9. Worthington, R.E., Boggess, T.S. Jr., and Heaton, E.K. (1972) *J. Fish. Res. Board Can. 29,* 113-115.
10. Worthington, R.E., and Miller, J. (1983) *Nutr. Rpts. Intern. 28,* 1361-1368.
11. Abell, L.L., Levy, B.B., and Kendall, F.E. (1952) *J. Biol. Chem. 195,* 357-365.
12. Mao, S.J.T., and Kottke, B.A. (1980) *Clin. Chem. 26,* 1369-1375.
13. Cohen, N.L., Reyes, P., Typpo, J.T., and Briggs, J.M. (1967) *J. Nutr. 91,* 482-488.

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ERRATA

In the paper "Cytotoxic Activity of the Thioether Phospholipid Analogue BM 41.440 in Primary Human Tumor Cultures" by Dieter B.J. Herrmann and Herbert A. Neumann, *Lipids 22,* 955–957 (1987), the formula of BM 41.440 given in Scheme 1 on page 955 is incorrect. The correct formula is shown on page 952.

In the paper "Phase I Trial of the Thioether Phospholipid Analogue BM 41.440 in Cancer Patients" by Dieter B.J. Herrmann, Herbert A. Neumann, Wolfgang E. Berdel, Manfred E. Heim, Michael Fromm, Dietmar Boerner and Uwe Bicker, *Lipids 22,* 962–966 (1987), the chemical structure of BM 41.440 on page 962 is in error. The substituent in position-2 should be methoxymethyl. The correct structure is shown on page 952.

Effects of Cholesterol and 25-Hydroxycholesterol on Smooth Muscle Cell and Endothelial Cell Growth[1]

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Auto-oxidation products of cholesterol may play a role in atherogenesis. In order to determine whether cholesterol or 25-hydroxycholesterol, a cholesterol auto-oxidation product, affected growth of vessel wall cells, sparse and confluent cultures of rabbit thoracic aorta smooth muscle cells and human umbilical vein endothelial cells were exposed to these compounds for 88 hr. The compounds were administered at 10^{-4}, 10^{-5}, 10^{-6} or 10^{-7} M in either ethanol or fetal bovine serum (FBS) vehicle. Cells were counted electronically, and the results were expressed as the percent growth in experimental vs control wells. Cholesterol did not inhibit cell growth under any experimental condition. 25-Hydroxycholesterol had the following effects: inhibited confluent smooth muscle cell growth at 10^{-4} M in ethanol vehicle only; inhibited sparse smooth muscle cell growth in a dose-related manner at 10^{-4}, 10^{-5} and 10^{-6} M in ethanol vehicle, but in FBS vehicle inhibited at only 10^{-4} and 10^{-5} M; inhibited confluent human umbilical vein endothelial cells at 10^{-4} M in ethanol vehicle only; and inhibited sparse human umbilical vein endothelial cell growth at 10^{-4} and 10^{-5} M in ethanol vehicle only. Thus, rabbit aortic smooth muscle cell growth was more sensitive to inhibition by 25-hydroxycholesterol than human umbilical vein endothelial cell growth was. ***Lipids 23,*** **85–88 (1988).**

Elevated levels of serum cholesterol have been associated with increased risk of developing atherosclerosis in man (1,2). Additionally, studies in rabbits have indicated that cholesterol and its oxygenated products enhance arterial lesion formation (3,4). Imai et al. (3) had demonstrated that USP-grade cholesterol contained contaminants that when concentrated and administered to rabbits by gavage, caused increased aortic smooth muscle cell death after 24 hr, compared with purified cholesterol. Further analysis showed that the principle active contaminants were 25-hydroxycholesterol and cholestane-3β,5α,6β-triol (4). These same compounds were reported to be cytotoxic to confluent cultured rabbit aortic smooth muscle cells at concentrations of 5×10^{-5} M to 1×10^{-4} M (5). Furthermore, scanning and transmission electron microscopic studies showed that when rabbits were given 25-hydroxycholesterol (2.5 mg/kg, intravenously) there were significantly more aortic lesions within 24 hr of injection in the 25-hydroxycholesterol groups than in the control group (6). Areas of endothelial cell damage and subendothelial edema were apparent (6). These studies have led to the suggestion that the oxidized derivatives of cholesterol, rather than cholesterol per se, are important in initiating atherosclerotic lesions (3–5,7).

While previous studies showed decreased cell growth in sparse cultures of L-cells (8), primary fetal liver cells and hepatoma cells (9) exposed to 25-hydroxycholesterol, these cell types are not involved in the atherosclerotic lesion. In the report concerning the effects of 25-hydroxycholesterol on cultured aortic smooth muscle cells by Peng et al. (5), only confluent cultures of smooth muscle cells were studied, and the cytotoxicity of 25-hydroxycholesterol was evaluated only by light microscopic estimation of the percentage of dead and dying cells.

The experiments reported here were initiated to determine in a quantitative fashion the effects of 25-hydroxycholesterol and cholesterol on in vitro growth of aortic smooth muscle cells (SMC) and endothelial cells (EC), two cell types that play major roles in the development of atherosclerotic lesions (10). To this end, both sparse (log phase) and confluent SMC and EC cultures were exposed to pure 25-hydroxycholesterol or recrystallized cholesterol. Sparse SMC cultures might approximate the conditions found as SMC begin to migrate from the media into the intima; confluent SMC cultures resemble the aortic media in that they are multilayered and relatively nonproliferative. Similarly, sparse EC cultures could be an approximate model for areas of intimal injury or denudation, while confluent EC cultures approximated the intact intima. EC derived from human thoracic aorta (HAEC) would have been ideal for these experiments; however, we have very limited access to normal adult human tissue. Therefore, we used EC derived from human umbilical vein (HUVEC). We occasionally have had access to HAEC for smaller experiments and have found similar patterns of response in the HAEC and HUVEC. The determination of cell numbers in control and treated cultures was the endpoint of these studies.

MATERIALS AND METHODS

Materials. All tissue culture materials, with the following exceptions, were purchased from Grand Island Biological Co. (Grand Island, NY): fetal bovine serum was obtained from Sterile Systems (Logan, UT), and human fibronectin and endothelial cell growth factor were from the laboratory of Robert Weinstein, Boston, MA. All culture vessels were from Falcon Plastics (Oxnard, CA). Factor VIII-related antigen was obtained from Atlantic Antibodies (Scarborough, ME). Heparin was from Sigma Chemical Co. (St. Louis, MO). Recrystallized cholesterol and pure 25-hydroxycholesterol were from the laboratory of John Partridge, Hoffmann-La Roche Inc. (Nutley, NJ).

Cells. Rabbit thoracic aorta SMC were obtained from a 15-week-old male New Zealand White rabbit by a modification of the explant technique of Tansik et al. (11). The thoracic aorta was removed under aseptic conditions and placed in ice-cold growth medium consisting of

[1]Part of this work was presented at the 10th Annual Hugh Lofland Conference on Arterial Wall Metabolism, Boston, MA, in 1985 and at the 70th Annual FASEB Meeting, St. Louis, MO, in 1986. It is published in abstract form (Fed. Proc. *45*;684, 1986).

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Abbreviations: ANOVA, analysis of variance; EC, endothelial cells; EDTA, ethylenediamine tetraacetic acid; FBS, fetal bovine serum; HAEC, human thoracic aorta endothelial cells; HUVEC, human umbilical vein endothelial cells; SEM, standard error of the mean; SMC, smooth muscle cells.

Dulbecco's Modified Eagle's Medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml Fungizone and 10% fetal bovine serum. The vessel was passed over a dry glass rod to remove endothelial cells. The adventitia was removed with forceps under a dissecting microscope. Specimens of tissue were saved and examined histologically with hematoxylin and eosin stain to verify the absence of adventitia and endothelial cell layers. Pieces of tissue ca. 1 mm^3 were immobilized under glass cover slips in 60 mm-diameter culture dishes. When the cells in the dish virtually were confluent, the cells were harvested using a trypsin and ethylenediamine tetraacetic acid (EDTA) solution and plated into 75-cm^2 tissue culture flasks. Three serial passages at 20-min intervals were performed to minimize contaminating fibroblast cells. The cells were passaged twice before being frozen. Cultures utilized in the study were at no greater passage number than four.

HUVEC were obtained from the laboratory of Robert Weinstein, Boston, MA. They were found to be free of mycoplasma contamination (12) and exhibited a positive immunofluorescent response to antihuman factor VIII-related antigen (13). Stocks of these cells routinely were maintained in tissue culture vessels treated with human fibronectin applied at 5–10 $\mu g/cm^2$. HUVEC were grown in Medium 199 with Earle's salts supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml Fungizone, 90 μg/ml heparin (16.0 U.S.P. units/ml) and 100 μg/ml endothelial cell growth factor.

Cholesterol and 25-hydroxycholesterol were dissolved in 95% ethanol. When fetal bovine serum without ethanol was used as a vehicle, sonication in an L&R transistor/ultrasonic T-28 sonicator at maximum output was employed to reduce particle size and aid solubility. Care was taken so that the samples were not heated.

Experimental design. Sparse and confluent cultures were grown in 35 mm dishes. Sparse cultures were seeded at 1.4×10^4 cells per dish in 2 ml of the appropriate growth medium supplemented with 10% fetal bovine serum (FBS). After six hr, this medium was aspirated and replaced with the appropriate media supplemented with experimental sterol, vehicle and FBS concentration. Cultures exposed to sterols at 10^{-4} M, 10^{-5} M, 10^{-6} M and 10^{-7} M with ethanol as the vehicle had final ethanol concentrations of 1%, 0.1%, 0.01% and 0.001%, respectively. All dishes treated with sterols were compared with control dishes that had received equivalent amounts of vehicle. After 88 hr of treatment, the cells were trypsinized and counted electronically using a Coulter counter. Confluent cultures were treated identically to sparse cultures except that experimental treatment began one day after the cells reached confluence. After 88 hr of treatment, these cultures were trypsinized and counted. Duplicate or triplicate plates were counted for each experiment. Doses of 10^{-7} M in SMC and 10^{-6} M in HUVEC were tested only once. All other doses were repeated two to five times. Results are expressed as the percent growth in experimental dishes compared with appropriate control dishes.

Statistical analysis. A cell means model, an analysis of variance (ANOVA) varient (14), was employed to determine treatment significance. Results are reported as percent growth in experimental dishes vs control dishes ± standard error of the mean (SEM).

RESULTS

Fetal bovine serum concentration effects. FBS concentrations of 10%, 5%, 2.5%, or 1% were tested to try detecting any cytoprotective effect of FBS in cultures containing 25-hydroxycholesterol. There was no consistent influence of FBS on the effect of sterols on sparse or confluent SMC or HUVEC cultures. Therefore, for correct statistical analysis results have been pooled across serum concentrations.

Effects of ethanol on SMC and HUVEC growth. Ethanol at final concentrations of 1.0%, 0.1% and 0.01% had no effect on sparse or confluent SMC growth (Table 1). Similar results were obtained with sparse and confluent HUVEC cells (Table 1) except that sparse HUVEC growth was decreased significantly to 75% of control ($p \leq 0.001$) by 1.0% ethanol (Table 2).

Effects of cholesterol on SMC and HUVEC growth. Cholesterol in either FBS or ethanol vehicle did not affect significantly the growth of sparse or confluent SMC (Table 3). However, a trend to increased cell growth (119% of control) regardless of vehicle was observed with sparse SMC incubated with 10^{-4} M cholesterol (Table 3). Cholesterol also had no significant effect on HUVEC growth except in ethanol vehicle at 10^{-6} M, which stimulated proliferation (121% of control) in the sparse HUVEC cultures ($p \leq 0.0001$) (Table 4).

TABLE 1

The Effect of Ethanol on Rabbit Aortic Smooth Muscle Cell Growth as Determined by Cell Number

% Ethanol	Confluent cultures[a]
1.0	90 ± 2
0.1	104 ± 3
	Sparse cultures[a]
1.0	92 ± 5
0.1	110 ± 6
0.01	102 ± 6

[a]The results are the percent of control growth ± SEM based on triplicate samples in one to five experiments after 88 hr of treatment.

TABLE 2

The Effect of Ethanol on Human Umbilical Vein Endothelial Cell Growth as Determined by Cell Number

% Ethanol	Confluent cultures[a]
1.0	92 ± 3
0.1	95 ± 3
	Sparse cultures[a]
1.0	75 ± 2*
0.1	96 ± 3
0.01	89 ± 3

[a]The results are the percent of control growth ± SEM based on triplicate samples in one to five experiments after 88 hr of treatment.
*P-value ≤ 0.0001.

TABLE 3

The Effects of Cholesterol and 25-Hydroxycholesterol on Rabbit Aortic Smooth Muscle Cell Growth as Determined by Cell Number

	Concentration	Confluent cultures[a]	
Sterol	M	Ethanol	FBS
Cholesterol	10^{-4}	96 ± 3	104 ± 5
	10^{-5}	97 ± 3	105 ± 3
	10^{-6}	102 ± 4	102 ± 3
	10^{-7}	—	103 ± 5
25-Hydroxycholesterol	10^{-4}	50 ± 2*	106 ± 6
	10^{-5}	93 ± 2	107 ± 3
	10^{-6}	107 ± 2	103 ± 3
	10^{-7}	—	105 ± 5
		Sparse cultures[a]	
		Ethanol	FBS
Cholesterol	10^{-4}	119 ± 9	119 ± 7
	10^{-5}	98 ± 4	92 ± 4
	10^{-6}	101 ± 3	93 ± 4
	10^{-7}	—	97 ± 2
25-Hydroxycholesterol	10^{-4}	29 ± 5*	68 ± 17*
	10^{-5}	32 ± 32*	52 ± 4*
	10^{-6}	72 ± 10*	97 ± 5
	10^{-7}	—	103 ± 2

[a]These results are the percent of control growth ± SEM based on duplicate samples in from one to five experiments after 88 hr of treatment.

*P-values ⩽ 0.0001.

TABLE 4

The Effects of Cholesterol and 25-Hydroxycholesterol on Human Umbilical Vein Endothelial Cell Growth as Determined by Cell Number

	Concentration	Confluent cultures[a]	
	M	Ethanol	FBS
Cholesterol	10^{-4}	89 ± 5	93 ± 4
25-Hydroxycholesterol	10^{-4}	52 ± 6*	96 ± 4
	10^{-5}	103 ± 3	—
		Sparse cultures[a]	
		Ethanol	FBS
Cholesterol	10^{-4}	103 ± 7	101 ± 4
	10^{-5}	97 ± 6	96 ± 8
	10^{-6}	121 ± 3*	99 ± 8
25-Hydroxycholesterol	10^{-4}	73 ± 6*	96 ± 9
	10^{-5}	73 ± 5*	99 ± 5
	10^{-6}	123 ± 8*	100 ± 8

[a]These results are the percent of control growth ± SEM based on duplicate samples in from one to five experiments after 88 hr of treatment.

*P-values ⩽ 0.0001.

Effects of 25-hydroxycholesterol on SMC and HUVEC growth. Confluent SMC were unaffected by 25-hydroxycholesterol in FBS vehicle (Table 3). However, when ethanol was the vehicle, 25-hydroxycholesterol at 10^{-4} M decreased the number of confluent SMC to 50% of the control value ($p \leqslant 0.0001$). This decrease was caused by cell death because the final number of cells in the treated wells was lower than the number of cells in the confluent cultures at the initiation of treatment. Sparse SMC growth was inhibited by 25-hydroxycholesterol in FBS vehicle to 68% of control at 10^{-4} M ($p \leqslant 0.0001$) and to 52% of control at 10^{-5} M ($p \leqslant 0.001$). When ethanol was the vehicle, sparse SMC growth also was inhibited in a dose response fashion by concentrations of 25-hydroxycholesterol from 10^{-4} to 10^{-6} M ($p \leqslant 0.0001$) (Table 3). When FBS was the vehicle, 25-hydroxycholesterol did not affect growth in sparse or confluent HUVEC cultures (Table 4). With ethanol as the vehicle, confluent HUVEC growth was decreased to 50% of control by 10^{-4} M 25-hydroxycholesterol ($p \leqslant 0.0001$). As with the SMC, this decreased cell number was lower than the number of cells at initiation of treatment, reflecting cell death rather than inhibition of growth. Growth in sparse cultures was decreased 27% at both 10^{-4} M ($p \leqslant 0.001$) and 10^{-5} M ($p \leqslant 0.0001$) 25-hydroxycholesterol (Table 4). Growth was stimulated to 123% of control only in sparse HUVEC cultures by 10^{-6} M 25-hydroxycholesterol in ethanol vehicle ($p \leqslant 0.001$) (Table 4).

DISCUSSION

Several investigators (3–5,7,15) have proposed that auto-oxidation products of cholesterol rather than cholesterol itself are injurious to the arterial wall and consequently play a role in atherogenesis. This hypothesis largely is based on experiments that showed by morphological criteria that cholesterol auto-oxidation products, not purified cholesterol, were toxic to vascular smooth muscle cells in vitro (5) and to the endothelium in vivo (6). In the present study, the effects of recrystallized cholesterol and 25-hydroxycholesterol on the growth of sparse and confluent rabbit aortic SMC and HUVEC were measured after 88 hr of treatment. The results of these studies indicated that FBS concentration did not influence the effects of the sterols in either cell type. Ethanol, the vehicle most commonly used for in vitro studies of this type, also had no effect on cell growth except that 1.0% ethanol significantly did inhibit sparse HUVEC growth. Peng et al. (5) had reported that 0.8% ethanol caused no obvious morphological evidence of cytotoxicity in confluent SMC after 24 hr. Therefore, our results indicate that the growth assay may be a more sensitive measure of ethanol effects in sparse HUVEC cultures. Recrystallized cholesterol did not affect cell growth under any condition except at 10^{-6} M in ethanol vehicle, which stimulated sparse HUVEC growth.

As had been reported for other cell types (8,16), 25-hydroxycholesterol was more effective at inhibiting growth in sparse than in confluent SMC and HUVEC cultures. Presumably, sparse cultures with cells in the log phase of growth require more cholesterol for membrane formation than do confluent cultures (17). Since 25-hydroxycholesterol decreases HMG-CoA reductase activity by suppressing gene transcription (18) and by

increasing enzyme degradation by interacting with the membrane-embedded portion of the enzyme (19,20), sparse cultures are more affected than are confluent cultures.

HUVEC cultures were less sensitive to the growth inhibitory effects of 25-hydroxycholesterol than were SMC cultures. This effect may be due in part to the fact that the population doubling time is four-fold faster in SMC than in HUVEC cultures (data not shown). Only when the vehicle was ethanol did 10^{-4} M 25-hydroxycholesterol decrease the number of cells in confluent HUVEC cultures. Additionally, sparse HUVEC cultures were more resistant to 25-hydroxycholesterol than were sparse SMC cultures. In FBS vehicle, 25-hydroxycholesterol did not inhibit growth in sparse HUVEC cultures, whereas in ethanol vehicle there was growth inhibition at both 10^{-4} M and 10^{-5} M (Table 4).

In vivo experiments meant to demonstrate the effects of 25-hydroxycholesterol on arterial vessels either have yielded conflicting results or the results are interpreted very differently from investigator to investigator. One reason for this confusion is that a mixture of oxidized cholesterol contains numerous different compounds and varies tremendously in its composition, depending on the circumstances. Higley et al. (21) found that rabbits fed diets supplemented with either cholesterol or a mixture of oxidized cholesterol products showed greater lesion development in the cholesterol-supplemented diet than in the oxidized cholesterol-supplemented diet. Krut (22) reported that oxidized sterols enhanced the solubility of pure cholesterol implanted in rats and concluded that the presence of oxidized sterols enhanced the clearance of cholesterol and, therefore, decreased the risk of atherosclerosis. On the other hand, Peng et al. (6) and Taylor and Peng (7), using scanning and transmission electron microscopy, showed that damaged aortic endothelial cells could be detected within 24 hr in rabbits given concentrates of cholestrol oxidation products either intravenously or by gastric gavage. Confounding the problem in deciding the importance of oxidized sterols in atherogenesis is the lack of knowledge of the normal source of these compounds in vivo. Foods containing oxidized sterols and auto-oxidation products of serum cholesterol are potential sources of these compounds. However, it is difficult to measure accurately the levels of oxidized sterols in food and in serum (23). Additionally, 25-hydroxycholesterol was not detected using isotope dilution mass spectrometry in the serum of eight normal human volunteers in a report by Javitt et al. (24), although 26-hydroxycholesterol, an enzymic product of cholesterol metabolism, was detected. Thus, it remains a matter of speculation as to whether vessel wall exposure to low levels of oxidized sterols over the long time period required for atherogenesis represents a significant risk factor.

The in vitro results presented in this report indicate that relatively high concentrations of 25-hydroxycholesterol are required to inhibit the growth or cause the death of cultured aortic SMC and HUVEC, even when administered in ethanol vehicle that enhances the ability of these compounds to penetrate cell membranes (25). These in vitro data and the data of others (5,8,16,26) demonstrate that 25-hydroxycholesterol inhibits growth in many types of cultured cells. How these in vitro systems can be used to predict in vivo effects is unclear.

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REFERENCES

1. Lipid Research Clinics Program (LRCP) (1984) *J. Am. Med. Assoc. 251,* 351-364.
2. Lipid Research Clinic Program (LRCP) (1984) *J. Am. Med. Assoc. 251,* 365-374.
3. Imai, H., Werthessen, N.T., Taylor, B., and Lee, K.T. (1976) *Arch. Pathol. Lab. Med. 100,* 565-572.
4. Peng, S.-K., Taylor, C.B., Tham, P., Werthessen, N.T., and Mikkelson, B. (1978) *Arch. Pathol. Lab. Med. 102,* 57-61.
5. Peng, S.-K., Taylor, C.B., Hill, J.C., and Morin, R.J. (1985) *Atherosclerosis 54,* 121-133.
6. Taylor, C.B., and Peng, S.-K. (1985) *J. Am. Oil Chem. Soc. 62,* 633.
7. Peng, S.-K., Tham, P., Taylor, C.B., and Mikkelson, B. (1979) *Am. J. Clin. Nutr. 32,* 1033-1042.
8. Chen, H.W., Kandutsch, A.A., and Waymouth, C. (1974) *Nature 251,* 419-421.
9. Kandutsch, A.A., and Chen, H.W. (1977) *J. Biol. Chem. 252,* 409-415.
10. Steinberg, D. (1983) *Atherosclerosis 3,* 283-301.
11. Tansik, R.L., Namm, D.H., and White, H.L. (1978) *Prostaglandins 15,* 399-408.
12. Chen, T.R. (1977) *Exp. Cell Res. 104,* 255-262.
13. Maciag, T., Stemerman, M.B., Kadish, J., and Weinstein, R. (1982) *J. Cell. Biol. 94,* 511-520.
14. Searle, S.R. (1971) in *Linear Models,* pp. 324-326, John Wiley and Sons Inc., New York, NY.
15. Peng, S.-K., Taylor, C.B., Mosbach, E.H., Huang, W.Y., Hill, J., and Mikkelson, B. (1982) *Atherosclerosis 41,* 395-402.
16. Brown, M.S., Dana, S.E., and Goldstein, J.L. (1974) *J. Biol. Chem. 349,* 789-796.
17. Chen, H.W. (1984) *Fed. Proc. 43,* 126-130.
18. Luskey, K.L., Faust, J.R., Chin, D.J., Brown, M.S., and Goldstein, J.L. (1983) *J. Biol. Chem. 258,* 8462-8469.
19. Gil, G., Faust, J.R., Chin, D.J., Goldstein, J.L., and Brown, M.S. (1985) *Cell 41,* 249-258.
20. Chin, D.L., Gil, G., Faust, J.R., Goldstein, J.L., Brown, M.S., and Luskey, K.L. (1985) *Mol. Cell. Biol. 5,* 634-641.
21. Higley, N.A., Beery, J.T., and Taylor, S.L. (1985) *J. Am. Oil Chem. Soc. 62,* 634.
22. Krut, L.H. (1985) *J. Am. Oil Chem. Soc. 62,* 634.
23. Park, S.W., and Addis, P.B. (1985) *J. Am. Oil Chem. Soc. 62,* 623.
24. Javitt, N.B., Kok, E., Burnstein, S., Cohen, B., and Kutscher, J. (1981) *J. Biol. Chem. 256,* 12644-12646.
25. Brown, M.S., and Goldstein, J.L. (1980) *J. Lipid Res. 21,* 505-517.
26. Kandutsch, A.A., Chen, H.W., and Heiniger, H.-J. (1978) *Science 201,* 498-501.

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Rat Neutrophil Function, and Leukotriene Generation in Essential Fatty Acid Deficiency

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Since the essential fatty acid linoleic acid is the precursor of arachidonic acid and thus of leukotriene B_4 (LTB_4), essential fatty acid deficiency (EFAD) may result in decreased synthesis of this stimulator of neutrophil granulocyte functions. Peritoneal and blood neutrophils from rats fed a diet with only 0.3% of energy requirements as linoleic acid and exhibiting biochemical evidence of EFAD showed substantial functional impairments compared to neutrophils from rats maintained on a diet with 3% of the energy requirement as linoleic acid. Oxidative burst activation (assessed by chemiluminescence), chemotaxis and aggregation were impaired upon stimulation with formylpeptides or the ionophore A23187. In contrast, these functions were intact on stimulation with exogenous LTB_4. Chemiluminescence was slightly but not significantly enhanced in EFAD rat neutrophils compared to controls when stimulated with phorbol myristate acetate (PMA). There were no differences between EFAD and control peritoneal neutrophils in the number of f-met-leu-phe (fMLP) receptors, or in their affinity for the ligand, assessed with fML(^{3}H)P. The fraction of responding cells also were similar, assessed with dichlorofluorescein diacetate fluorescence. Moreover, the endogenous LTB_4production in response to A23187 or fMLP was decreased by 57.7% and 63.5%, respectively, in EFAD peritoneal neutrophils. Thus, EFAD was associated with reductions of LTB_4 production and neutrophil responsiveness to A23187 and formylpeptides but not to LTB_4 or PMA, which supports the hypothesis that endogenous LTB_4 may contribute to the activation of neutrophil functions involved in inflammation and host defense.
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Humans and many higher vertebrates are incapable of synthesizing linoleic acid (18:2n-6) (1) that consequently is an essential dietary requirement. Linoleic acid is metabolized by stepwise desaturation and elongation to arachidonic acid (AA, 20:4n-6), the obligate precursor to cyclooxygenase products, e.g. prostaglandins and thromboxanes of the 2-series and lipoxygenase products, e.g. the leukotrienes of the 4-series (2).

In neutrophils, the 5-lipoxygenase pathway predominates and one major product is 5(S),12(R)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid, leukotriene B_4 (LTB_4), formed by a hydrolase from the unstable allylic epoxide intermediate, 5(S)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, leukotriene A_4 (LTA_4) (2). LTB_4 production substantially is increased in activated neutrophils, e.g. after in vitro stimulation with the ionophore A23187 (3,4), opsonized zymosan (5) or formyl peptides (2,6). Exogenous LTB_4 is a potent inducer of neutrophil adherence (7), chemotaxis (8) and aggregation (9). Neutrophil degranulation (9) and oxidative burst activity, assessed by chemiluminescence (CL) (10) or superoxide anion production (9,11), also are initiated by LTB_4. Based on these and other observations, LTB_4 has been proposed as a putative endogenous mediator of neutrophil function in inflammation and host defense (10).

Essential fatty acid deficiency (EFAD) has been associated with increased susceptibility to bacterial infections (12) and altered inflammatory responses (13,14). Such observations might be explained partly by impaired leukocyte function following diminished production of lipid mediators from arachidonic acid. EFAD is associated with a decrease in arachidonic acid content of neutrophils and reduced production of cyclooxygenase products, as reported previously by us (15) and others (16). Furthermore, it recently was reported that leukotriene production in rat neutrophils was depressed in EFAD (17).

Based on these considerations, the aim of this study was to assess whether neutrophil functions contributing to inflammatory response and host defense such as the oxidative burst activation, chemotaxis, aggregation and leukotriene generation were altered in rats with a dietarily induced EFAD. These studies could help to clarify the role of leukotrienes in the stimulation of neutrophil functions.

EXPERIMENTAL

Animals and diets. Two groups of male, adult Sprague-Dawley rats, maintained for several generations on diets differing in EFA content, were used for this study. Details of the animals, the diets, the EFAD state and nutritional status have been given previously (15,18).

Chemicals. Hanks' balanced salt solution (HBSS) was obtained from SBL (Stockholm, Sweden). Luminol (10), human serum albumin (HSA, essentially fatty acid free), phorbol myristate acetate (PMA) and sodium caseinate was obtained from Sigma Chemical Co. (St. Louis, MO). Dichlorofluorescein diacetate (DCHF-DA) was from Eastman-Kodak (Rochester, NY). Ficoll-Paque was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-leucine (fNLPNTL), formyl-methionyl-leucyl-phenylalanine (fMLP) and formyl-methionyl-methionyl-methionyl-methionine (fMMMM) were from Peninsula Laboratory (San Carlos, CA). Synthetic leukotriene B_4 (LTB_4), dissolved in ethanol, was a gift from J. Pike (Upjohn Co., Kalamazoo, MI) and J. Rokach (Merck-Frosst, Dorval, Canada). The ionophore A23187 was from Calbiochem (LaJolla, CA). FML(3H)P (46.7 Ci/mmol) was from New England Nuclear (Boston, MA), Versilube F50

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Abbreviations: AA, arachidonic acid; CL, chemiluminescence; DCHF-DA, dichlorofluorescein diacetate; diHETE, dihydroxyeicosatetraenoic acid; EFA, essential fatty acid; EFAD, essential fatty acid; fMLP, N-formyl-methionyl-leucyl-phenylalanine; fMMMM, formyl-methonyl-methionyl-methionyl-methionyl-methionine; fNLPNTL, formyl-norleucyl-leucyl-phenylalanine-norleucy-tyrosyl-leucine; HBSS, Hanks' balanced salt solution; HETE, hydroxytetraenoic acid; HSA, human serum albumin; LTA, leukotriene A; LTB, leukotrine B; PMA, phorbol myristate acetate.

silicon fluid from General Electric Co. (Waterford, NY) and NCS tissue solubilizer from Amersham Corp. (Amersham, U.K.).

Preparation of neutrophils. Rats were anesthetized with ether by inhalation. Neutrophils were harvested from peritoneal cavities 22 hr after injection of sterile sodium caseinate (12 mg/ml) by peritoneal lavage with HBSS. The peritoneal exudates were purified further on Ficoll-Paque. Blood was obtained by decapitation of ether-anesthetized rats, and leukocytes were purified by a standard Ficoll-Paque technique (10). Peritoneal neutrophil preparations routinely were of 98% purity after purification and were suspended in HBSS with or without HSA, as indicated. Although the total yield of peritoneal cells was dependent on the amount of HBSS used in the lavage procedure, there were no consistent differences between the dietary groups. Typically, 80–100 $\times$ 10^6 neutrophils were obtained in technically successful lavages, the variation being from day to day rather than between dietary groups.

Due to paucity of animals and blood and the small amounts of neutrophils obtained from blood, peritoneal neutrophils were used in all assays except where specifically indicated.

Using the assays for functional responses described below, we assessed whether the peritoneal cell harvesting technique had activated the neutrophils. The spontaneous chemiluminescence was <5 mV, which is within the limits for unstimulated rat blood neutrophils. Although spontaneous aggregation was minimal for peritoneal cells, these migrated slightly farther than blood cells in absence of stimuli, thus activation of neutrophil functional responses could not be demonstrated consistently but cannot be excluded.

As reported, the peritoneal neutrophils from EFAD rats contained less linoleic acid and arachidonic acid than the control rats (15) and the relation of 20:3n-9/20:4n-6 was 0.42 (15), which demonstrates the EFAD state (19). In the control rat peritoneal neutrophils, 20:3n-9 was not measurable (15). The details of these analyses and of reduced generation of cyclooxygenase products in EFAD rat peritoneal neutrophils have been reported (15).

Eicosanoid analysis. Rat peritoneal neutrophils were preincubated five min at 37 C in HBSS (without HSA) at the concentration of 2 or 5 $\times$ 10^6/ml. The cells then were stimulated with 2 μM ionophore A23187, 1 μM FMLP or 10 μM PMA; after five min (A23187, PMA) or 30 min (fMLP (20), PMA) of incubation at 37 C, reactions were stopped by addition of an equal volume (1 ml) of methanol, and the samples were kept at -70 C.

Analysis of arachidonic acid metabolites was performed by reversed phase HPLC using a C_{18} Radial-Pak cartridge (100 $\times$ 8 mm ID, 10 μm particle size, Waters Associates, Milford, MA) protected by a C_{18} Guard Pak Cartridge (Waters Associates) as reported (21,22). The solvent system used has been described (22). PGB_3 (100 ng) was added to the methanol denatured samples as an internal standard, and these were centrifuged at 600 $\times$ g for 10 min. Supernatants were acidified and injected (injection volume, 1.8 ml) without further treatment (23).

Elution of the various compounds was monitored by UV photometry at 280 nm (LTs and PGB_2) and 229 nm (5-HETE). Reacylated 5-HETE was not quantitated. Identification of lipoxygenase products was assessed on the basis of their comigration with known standards and specificity of UV absorption at 280 or 229 nm. The quantification of LTs and 5-HETE was carried out by comparison of their peak areas with that of internal standard (PGB_2) and correction for differences in molar extinction coefficients and attenuation settings. The system used permits differentiation between 20-OH- and 20-COOH-LTB_4. Sensitivity of the system is 2–5 ng (21) for the different lipoxygenase products.

Functional studies. Chemiluminescence augmented by luminol was assessed with luminometers (1250 and 1251; LKB, Bromma, Sweden) essentially as detailed previously (10). Each 1 ml sample contained luminol (final concentration 0.17mM), 1.25 $\times$ 10^6 neutrophils and HBSS with 0.2% HSA. When A23187 was used as stimulus, HSA was omitted since albumin impaired its CL response. CL was read continuously for up to 15 min. Neutrophils were kept at 4 C and adjusted to 37 C prior to analysis. Stimuli used were fMMMM (1 μM), fNLPNTL (1 μM), A23187 (2 μM), PMA (10 μM) and LTB_4 (1 μM). Blood neutrophils were assessed in two experiments using fMMMM (1 μM), or PMA (10 μM). Due to the limited availability of blood neutrophils, these assessments were scaled down to 0.25 ml with preserved component ratios. In preliminary experiments, these concentrations of stimuli had been found optimal for assessment of chemiluminescence. fMLP was used in a prior study (15); however, the responses to fMMMM and fNLPNTL were found to be more consistent in the CL assay.

Neutrophil aggregation was assessed in a standard platelet aggregometer (model 300BD, Payton Assoc., Buffalo, NY) (23). An aliquot of 0.45 ml of the neutrophil suspension (10 $\times$ 10^6 neutrophil/ml) was added to a siliconized cuvette, containing a stir bar revolving at 900 rpm. After equilibration of the cells for two min, 50 μl of the stimulus (1–10 μM ionophore A23187 or 0.01–1 μM LTB_4) was added. The resulting change in light transmission was recorded as Δ T. Results for LTB_4 and fMLP are expressed as the maximal change in light transmission (peak response), time to peak and degree of desaggregation one min after peak, and for A23187 as Δ T three min after addition of stimulus because A23187 induced an irreversible aggregation response (24).

Chemotaxis. Spontaneous and stimulated migration was assessed with a modified Boyden chamber technique, using a 48 multiwell chamber (Neuroprobe Inc., Cabin John, MD). Cells, in HBSS supplemented with 0.4% HSA, were allowed to migrate into cellulose nitrate filters (Microfiltration Systems, Dublin, CA; 3 μm pore diameter) for 45 min at 37 C, 100% humidity and 5% CO_2. fMLP and LTB_4 were used as chemotactic factors and HBSS as control for spontaneous migration. Migration was assessed as the mean depth in micrometers of penetration into the filter of leading five cells in three microscopic fields in three replicate wells, and results are given as net migration (stimulated migration minus spontaneous migration, i.e. towards HBSS). Optimal migration for control peritoneal neutrophils occurred at 1–0.1 μM of fMLP and 0.1 μM LTB_4.

fML(^{3}H)P-binding was performed on ice as described (25). Briefly, fML(^{3}H)P only (total binding) or fML(^{3}H)P plus a 1,000-fold excess of nonradioactive fMLP (nonspecific binding) were added to suspensions of rat peritoneal neutrophils (5 $\times$ 10^6/ml in HBSS without

calcium and magnesium). In addition, at the end of the 30-min incubation an aliquot of cells from total binding tube was added to 1,000-fold excess nonradioactive fMLP to allow the surface-bound fML(^{3}H)P to be displaced off the cells (displaceable binding). The bound and free peptide were separated by centrifugation of cells through silicon oil. The difference between total and nonspecific binding of fML(^{3}H)P represented total specific binding of fML(^{3}H)P. By using the specific binding data at varying concentrations of fML(^{3}H)P, a Scatchard plot was performed (26).

Dichlorofluorescein fluorescence. Since neutrophil populations are inhomogenous in regard to the binding of fMLP (27), the fraction of responding cells (recruitment) was assessed. The H_2O_2 dependent DCHF-DA fluorescence (28) (not requiring phagocytosis) was assessed by fluorescence microscopy. Peritoneal neutrophils (1×10^6/ml) were incubated with DCHF-DA (10 μg/ml) for five min in HBSS without Ca^{2+} and Mg^{2+} (to minimize preactivation) at 37 C, pelleted at 200 × g and washed with HBSS. After resuspension in HBSS with Ca^{2+} and Mg^{2+}, neutrophils were stimulated with fMLP (0.1 or 1 μM), LTB_4 (0.1 μM) or A23187 (2 μM), concentrations previously found optimal, or HBSS (negative control) for five min (fMLP) or 20 min (A23187, PMA). The reaction was stopped on ice, and glutaraldehyde (2% v/v) was added. The cells were washed and resuspended in HBSS and studied under high power magnification in an Olympus fluorescence microscope with a reflected fluorescence light source. For each condition, 500 cells were counted in triplicate. The weak background fluorescence repeatedly was correlated with the unstimulated cells.

Statistical analysis was performed with Student's two-tailed t-test.

RESULTS

We studied the lipoxygenase product synthesis in neutrophils after stimulation with the ionophore A23187. Cells from normal rats released LTB_4, 20-COOH-LTB_4, 5-HETE as well as the products of nonenzymatic hydrolysis of LTA4, e.g., the 6-trans-12-epi-LTB_4, 6-trans-LTB_4 and the 5,6-DiHETEs. Neutrophils from EFAD rats produced the same metabolites but in significantly lower quantities. As shown in Figure 1, the levels of LTB_4, 20-COOH-LTB_4, 5-HETE and 6-trans-LTB_4 were decreased by 57.7 ± 1.2%, 39.6% ± 9.8%, 63.1 ± 9.6% and 26.4 ± 8.9%, respectively, compared to the control neutrophils (mean ± S.E.). LTB_4 production following fMLP stimulation was substantially lower than the A23187 induced formation being 2.7 ± 0.7 ng/10^6 neutrophils for the control animal cells. The EFAD neutrophils showed a decrease of LTB_4 production by 60.4 ± 6% (±SE, n = 3, $p < 0.01$). 20-COOH-LTB_4 was not detectable in EFAD or control neutrophils on fMLP stimulation. PMA stimulation did not induce any LTB_4 production. The peptidoleukotrienes LTB_4, D_4 and E_4, as well as 20-OH-LTB_4, were not detectable for any stimulus. Since both 20-OH- and 20-COOH-LTB_4 easily can be detected in the present HPLC system, a rapid turnover of 20-OH-LTB_4 to 20-COOH-LTB_4 is likely, preventing its accumulation and detection.

Given these data on endogenous LTB_4 production, we assessed neutrophil functional responses to see if there was a relation to endogenous LTB_4 production.

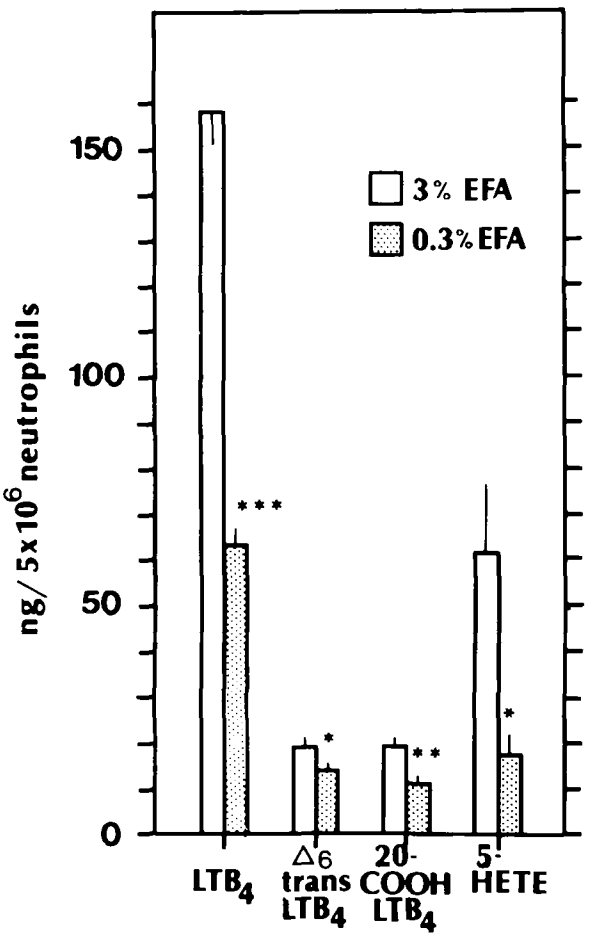

FIG. 1. Production of leukotrienes and 5-HETE in neutrophils from EFAD and control rats. Peritoneal neutrophils (5×10^6/ml) were stimulated with the ionophore A23187 (2 μM), analysis was performed by reversed phase HPLC as detailed in the Methods section. Results are given as ng/5×10^6 neutrophils (±SE, n = 4-5). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

For assessment of oxidative burst activity, luminol augmented CL was applied. The CL responses from EFAD rats and controls were identical kinetically, and there was no difference in spontaneous CL. The response to formylpeptides showed a small initial peak with a duration of seconds, immediately continuing in a slightly more protracted major peak without returning to baseline values in between. All calculations were made from the second, major peak. The response to LTB_4 was of lesser magnitude than the response to formylpeptides and single-peaked, thus resembling the response from human blood neutrophils (10). A23187 and PMA-induced CL appeared considerably slower than for the other stimuli. This also is in accordance with findings for human neutrophils (10).

The responses to A23187 and the formylpeptides fMMMM and fNLPNTL significantly were diminished in the EFAD neutrophils compared with the controls (Fig. 2). In contrast, the CL response of EFAD cells to exogenous LTB_4 and PMA were slightly but not significantly increased as compared with the controls (Fig. 2). The CL generated by EFAD blood cells after stimulation with FMMMM was 4.6 ± 1.2 mV compared to 20.3 ±

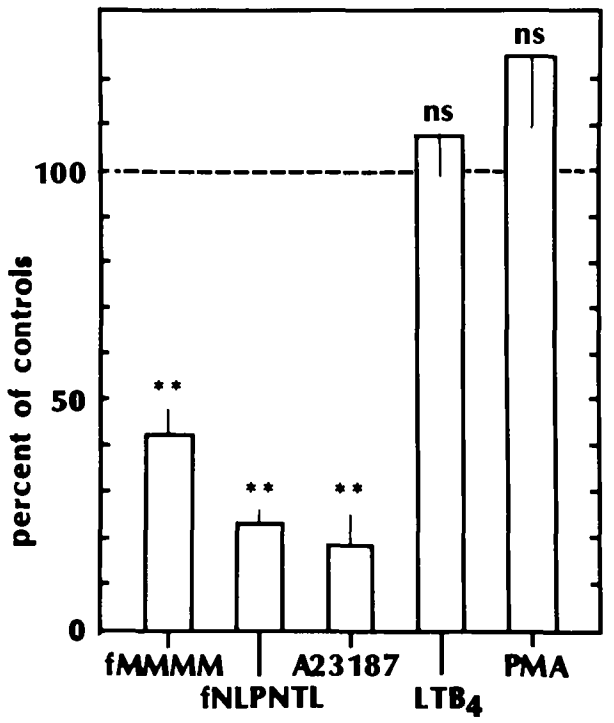

FIG. 2. Chemiluminescence (CL) augmented by luminol, produced by peritoneal neutrophils (1.25×10^6/ml) from EFAD rats expressed as percent of CL generated by neutrophils from control rats. CL was induced by the formylpeptides fMMMM and fNLPNTL (both 1 μM), A23187 (2 μM), leukotriene B4 (LTB_4, 1 μM) and phorbol myristate acetate (PMA, 10 μM). CL generated by control rat neutrophils stimulated by the different stimuli was fMMMM, 19.4 ± 1.1 mV; fNLPNTL, 12.9 ± 2 mV; A23187, 31.4 ± 4.2 mV; LTB_4, 2.5 ± 0.5 mV; PMA, 69.4 ± 3 mV (± S.E., n = 5-7). **, $p < 0.01$; ns, not statistically significant ($p > 0.05$).

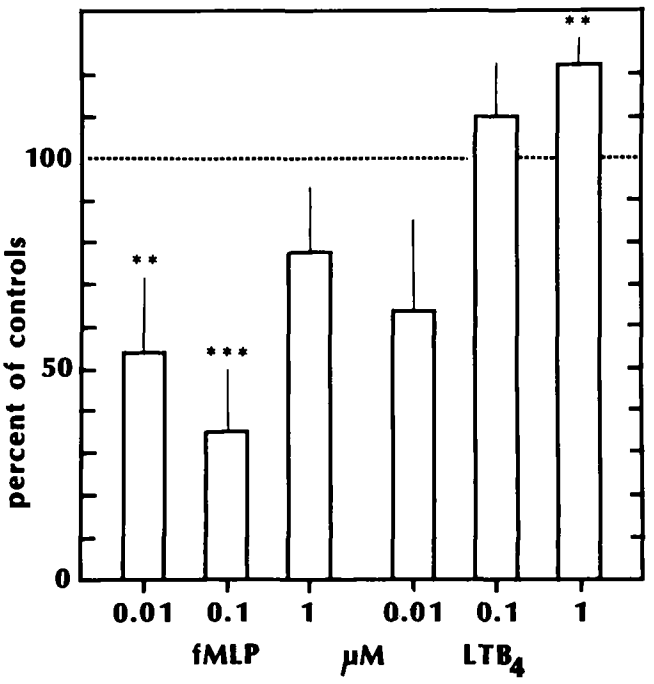

FIG. 3. Net migration (stimulated minus spontaneous migration) assessed as the leading front of neutrophils that were obtained from EFAD rats after stimulation with fMLP or LTB_4. Results are expressed as mean and S.E. values of simultaneously run controls (neutrophils from rats maintained on a diet with 3% linoleic acid). The distances migrated by control cells were for spontaneous migration 50+/−7 μm and for fMLP at 1 μM, 26+/−9, at 0.1 μM, 23+/−5, and at 0.01 μM 19+/− μM (net migration). The corresponding values for LTB_4 were 23+/−11, 38+/−6 and 26+/−6 μm. *, $p \oplus 0.05$; *, $p < 0.01$; ***, $p < 0.005$.

1.2 mV by the controls ($n = 2$, $p < 0.05$). When PMA (10 μM) was used as stimulus, EFAD blood cells responded with 26.1 ± 3.3 mV and controls with 29.3 ± 0.4 mV ($n = 2$, $p = 0.3$).

We next assessed neutrophil migration and aggregation. The spontaneous migratory capacity of peritoneal cells from EFAD rats was slightly but not significantly higher (118 ± 7%) compared to the controls ($p > 0.05$). Rat peritoneal neutrophils responded with a stimulated migration to both fMLP and, contrary to previous reports (24,29), LTB_4. When fMLP was used as chemoattractant, EFAD cells did not migrate as far as controls (Fig. 3). This difference was highly significant at fMLP concentrations of 0.1 μM ($p < 0.005$) and 0.01 μM ($p < 0.01$) but not significant at 1 μM. In contrast, no difference was observed when LTB_4 was used as chemoattractant, except at 1 μM where a small but significant ($p < 0.01$) enhancement was noted (Fig. 3). Chemotaxis of blood neutrophils also was assessed. Optimal migration was observed towards 0.1 μM fMLP. EFAD cells migrated only 46% of the distance of control rat cells (Table 1). When LTB_4 was used as stimulus, very small increments of migration was observed (in accordance with 24,29), being approximately 1/5-1/8 of what human neutrophils exhibit at optimal stimulus concentration (i.e. 10 nM). However, there was no difference between the dietary groups. Thus, the results from the experiments on blood neutrophils were similar to what was observed for peritoneal cells.

TABLE 1

Chemotaxis of Rat Blood Neutrophils

		fMLP, μM		LTB_4, μM	
		0.01	0.1	0.01	0.1
EFAD, μm	20 ± 7	21 ± 2	46 ± 13	12 ± 1	14 ± 1
Controls, μm	20 ± 4	47 ± 8	78 ± 4	12 ± 2	17 ± 3

Mean and SE for two separate experiments. In each experiment, triplicate analyses for each condition was performed. In addition to the stimulus concentrations given above, experiments were run with fMLP and LTB_4 at 1 nM and 1 μM; only small net increments of migration were observed in these experiments. Values given for fMLP and LTB_4 represent net migration.

The propensity to aggregate also was changed. Ionophore A23187 induced a continuous aggregation not followed by any desaggregation. The aggregation of EFAD peritoneal neutrophils was depressed at all tested A23187 concentrations and significantly at 1-10 μM ($p < 0.05$; Fig. 4). The kinetics of aggregation for control and EFAD cells were similar. Control neutrophils responded

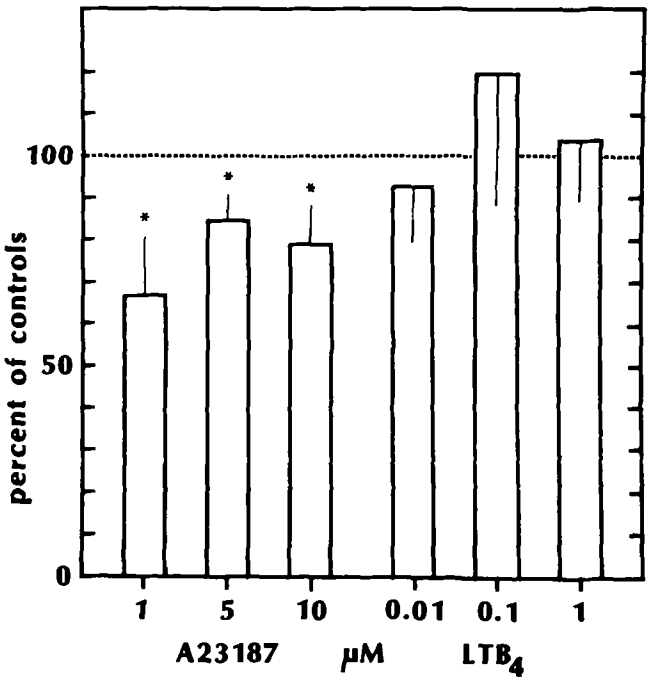

FIG. 4. Aggregation of EFAD neutrophils after stimulation with ionophore A23187 (1–10 μM; n = 5–9) and LTB_4 (0.1–10 μM; n = 5–9). The recorded change in light transmission (ΔT in mm) was measured three min after addition of A23187 and at peak for LTB_4. The results are expressed as mean and S.E. percent values of controls. *, $p < 0.05$.

to 1 μM LTB_4 stimulation with a peak after 40 sec and desaggregated with 35% of the peak value one min after the peak. The peak response to LTB_4 in EFAD rat neutrophils was of the same magnitude as to control cells, and desaggregation was not significantly different. However, the time to peak was delayed at all tested concentrations, and at 1 μM the peak was delayed by 16 sec ($p < 0.01$) (data not shown). As previously reported fMLP (0.1 μM) induced aggregation was depressed in EFAD compared to control animals (15).

It previously has been shown that chemoattractant receptor affinity is regulated by its lipid environment (30). As alteration of membrane lipid fluidity is conceivable in EFAD and thus may modulate receptor binding, we determined the fMLP (^{3}H)P binding to its receptors on peritoneal neutrophils from the differently fed rats.

In each of three experiments, there was no difference in total specific binding between the rats (Fig. 5) nor in displaceable or nondisplaceable binding.

Using Scatchard analysis, it was evident that the binding plots obtained were curvolinear for both dietary groups but also could fit two different linear functions calculated using linear regression analysis (plot A and plot B in Fig. 5), assuming a two-receptor model with different affinities. Figure 5 shows that the slope of plot A, a measure of binding affinity (slope = $-1/Kd = -Ka$) was similar for the EFAD and control rat cells (0.36 ± 0.16 vs 0.39 ± 0.10, respectively) and there was no difference between the slopes of data fitted by B-lines (−0.08 ± 0.02 vs −0.04 ± 0.1).

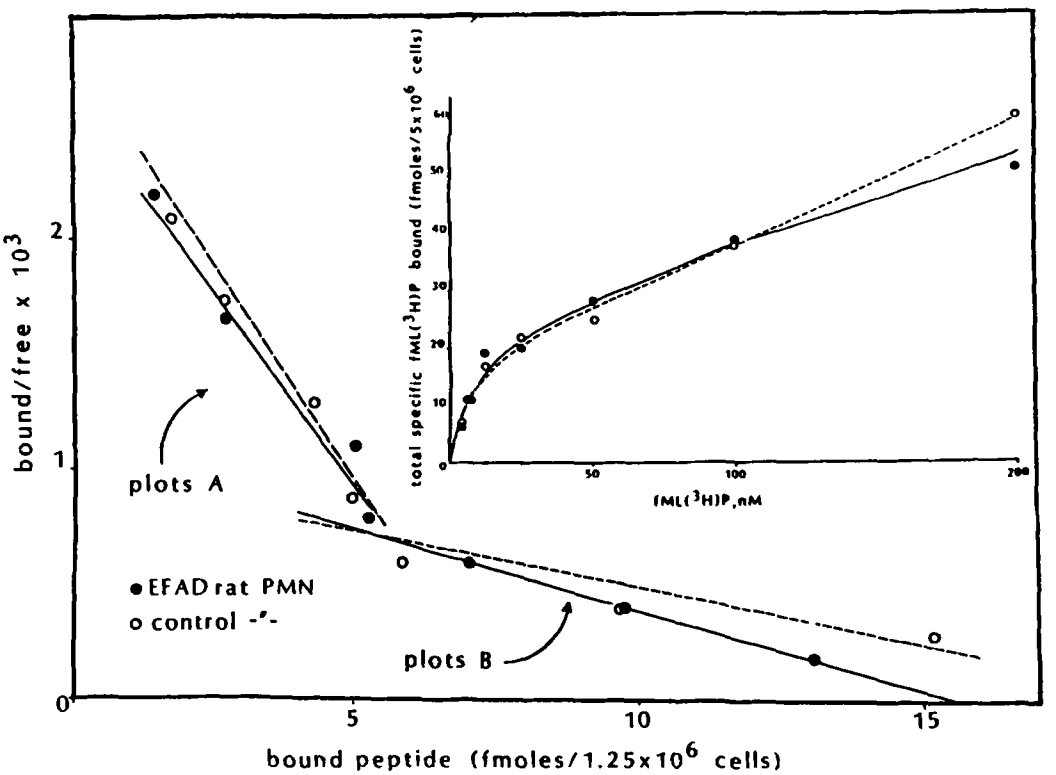

FIG. 5. Scatchard analysis of fML(^{3}H)P binding to peritoneal neutrophils from EFAD and control rats. The lines drawn are calculated using linear regression analysis. Points represent mean of three experiments. Insert, total specific fML(^{3}H)P binding to neutrophils from EFAD and control rats after 30 min incubation with increasing doses of fML(^{3}H)P at 4 C. Points represent mean of three experiments.

In these experiments, the number of binding sites per cell were 5725 ± 928 and 7000 ± 1427 (n = 3) for EFAD and control rat neutrophils, respectively. The small difference in receptor number was not statistically significant.

Finally, by studying the fraction of responding peritoneal neutrophils with DCHF-DA fluorescence, It was evident that the differences in CL between EFAD and control rat cells upon fMLP or A23187 stimulation were not due to a decreased fraction of responsive neutrophils (Table 2).

TABLE 2

Dichlorofluoresceindiacetat Fluorescence

Stimulus	Concentration μM	Percent responding cells	
		EFAD	Control
fMLP	0.1	76 ± 6.4	80 ± 3.3
fMLP	1.0	79.4 ± 2.2	81.7 ± 2.1
LTB_4	0.1	76.1 ± 1.5	79.2 ± 2.1
A23187	2.0	78.3 ± 1.8	80.3 ± 1.8
PMA	10.0	84.9 ± 5.8	84.8 ± 2.3

Performed as detailed in the Experimental section. Results are given as the fraction of responding (fluorescent) cells.
Mean ± SE, n = 3.

DISCUSSION

We previously have reported the fatty acid composition of these EFAD rat peritoneal nuetrophils (15). The decrease in cellular arachidonic acid content was 33%. However, in this study the production of some lipoxygenase metabolites were reduced by 60–70%, thus substantially lower than what would be expected from the arachidonic acid deficiency per se (Fig. 1) and in accordance with another report (17). The mechanism for this disproportionate 5-lipoxygenase product deficiency in EFAD neutrophils might be related to the presence in the test cells of the arachidonic acid analogue 20:3n-9 as suggested previously (17). This trienoic fatty acid, a derivative of oleic acid, is undetectable or present in only minor quantities in normal neutrophils but increased in EFAD (12,19) and significantly so in neutrophils from EFAD rats in this study (15). It is noteworthy that the irreversible inhibition of LTA_4 hydrolase by the 20:3n-9 metabolite, 5(6)-oxido-7,9,11-(E,E,Z)-eicosatrienoic acid (leukotriene A_3), reported recently (17), cannot account for the reduction of 5-lipoxygenase product synthesis reported herein since the formation of 5-HETE on A23187 stimulation, which occurs at a step above the LTA_4 hydrolase, also was inhibited. Clearly, further studies are required to clarify this issue.

LTB_4 has been postulated to be a mediator of neutrophil functions. The rapid generation of leukotrienes following stimulation with a variety of stimuli (2–6), the activation of neutrophil functional responses by LTB_4 (7–11) and to a lesser extent, the inhibiting effects of lipoxygenase inhibitors (4,11,31–33) constitute circumstantial evidence for a role of endogenous LTB_4. The EFAD neutrophil model offers an additional means to study the physiological significance of lipoxygenase products in neutrophil function.

In EFAD neutrophils, in which a defect of 5-lipoxygenase product synthesis has been shown, there also were functional impairments to stimuli that induce leukotriene synthesis, e.g. to A23187, which has potent and prompt leukotriene generation promoting ability (3,4). Responses to formylpeptides, which are weaker stimuli for leukotriene generation (2,6), also were affected. Thus, it is conceivable that full functional responsiveness may require unaltered leukotriene production. Finally, the noted impairments did not appear to be due to a general alteration of the EFAD neutrophil responsiveness since spontaneous migration and spontaneous CL both were intact in EFAD neutrophils. Furthermore, the response to exogenous LTB_4 (34) or PMA, which does not induce LTB_4 generation (35, and as shown here), was equal to controls, showing that the EFAD neutrophils retained full capacity to respond to stimulation.

The differences observed between EFAD and control neutrophils after stimulation with certain agents was similar in blood and peritoneal cells and indicated a discrete defect of the stimulus-response coupling. One explanation might be decreased membrane fluidity due to the altered fatty acid composition of membrane phospholipids, which can be of particular consequence for complex functions, e.g. chemotactic and aggregatory responsiveness. However, it previously has been shown that moderate EFAD in rats does not alter erythrocyte membrane fluidity to any appreciable degree (36). Also, a simple relation seems not to exist between cell membrane fluidity and EFA composition of the diet (37,38). Furthermore, responses to LTB_4 in this study showed that the chemotactic and aggregatory potential in response to a membrane receptor dependent stimulus, per se, was unaffected in EFAD peritoneal neutrophils.

Another explanation for the observed decrements of EFAD neutrophil responses could relate to previous observations that alterations of cell membrane fatty acid composition may be associated with altered surface receptor expression (16), e.g. due to receptor numbers and/or functional state. The normal responsiveness to exogenous LTB_4 argues against a general explanation based on reduced receptor numbers in the EFAD peritoneal neutrophils. However, the most pronounced reduction in activity of EFAD neutrophils was observed on stimulation with A23187, which does not depend on a receptor for activation.

The present data show that the fMLP-receptor on rat peritoneal neutrophils has high- and low-affinity states but that the receptor affinity and the receptor number were similar for EFAD and control rats. Thus, differences as to responses to fMLP between the groups were not due to receptor expression changes.

The data reported in this study support the concept that deficiency of endogenous LTB_4 may be related to the functional impairment in the EFAD neutrophils, a defect that can be overcome by addition of exogenous LTB_4. Given the reported biological activities of LTB_4, it is possible that the LTB_4 generated in the neutrophils upon stimulation, e.g. by fMLP, contributes to the effect of the given stimulus on the cells. It also is clear from previous studies that this LTB_4 cannot account for all of the neutrophil response; Sha'afi et al. have suggested that

fMLP can activate rat neutrophils through mechanisms independent of LTB_4 generation (39).

The results presented here show a striking similarity to what is to be reported from another study on the effects of EFAD on neutrophils, in which EFAD was induced by fat-free total parenteral nutrition (40). In that condition, EFAD develops rapidly, probably because a glucose-dependent hyperinsulinemia impedes mobilization of linoleic acid from adipose tissue. Regardless of the model of induction, it is apparent that several functional responses such as chemotaxis, aggregation, oxidative burst activation and degranulation (19,31) can be impaired. Such reductions may be significant when looking for explanations of EFAD-associated inflammatory dysfunctions and increased susceptibility to bacterial infections. EFAD finally may be clinically more important and prevalent than previously believed since it may occur as part of protein-energy undernutrition (41) because of deficiency of desaturating and elongating enzyme systems. Considering that LTB_4 is a potent activator of neutrophils, its reduced synthesis may cause impaired host defense in individuals with no overt, clinical signs of malnutrition.

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REFERENCES

1. Holman, R.T. (1971) *Progr. Chem. Fats Other Lipids 9,* 275-348.
2. Jubiz, W., Radmark, O., Malmsten, C., Hansson, G., Lindgren, J.Å., Palmblad, J., Uden, A.M., and Samuelsson, B. (1982) *J. Biol. Chem. 257,* 6106-6110.
3. Borgeat, P., and Samuelsson, B. (1979) *J. Biol. Chem. 254,* 7865-7869.
4. Samuelsson, B. (1983) *Science 220,* 568-575.
5. Claesson, H.E., Lundberg, U., and Malmsten, C. (1981) *Biochem. Biophys. Res. Commun. 99,* 1230-1237.
6. Ham, E.A., Söderman, D.D., Zanetti, M.E., Dougherty, H.W., McCauley, E., and Kuehl, P.A. (1983) *Proc. Natl. Acad. Sci. USA 80,* 4349-4353.
7. Dahlen, S.E., Björk, J., and Hedqvist, P. (1981) *Proc. Natl. Acad. Sci. USA 78,* 3887-3992.
8. Malmsten, C., Palmblad, J., Uden, A.M., Radmark, O., Engstedt, L., and Samuelsson, B. (1980) *Acta Physiol. Scand. 110,* 449-451.
9. Serhan, C.N., Radin, A., Smolen, J.E., Korchak, H., Samuelsson, B., and Weissmann, G. (1982) *Biochem. Biophys. Res. Commun. 107,* 1006-1011.
10. Palmblad, J., Gyllenhammar, H., Lindgren, J.Å., and Malmsten, C.L. (1984) *J. Immunol. 132,* 3041-3045.
11. Sumimoto, H., Koichiro, K., and Minakami, S. (1984) *Biochim. Biophys. Acta 803,* 271-277.
12. Holman, R.T. (1978) in *CRC Handbook Series in Food and Nutrition* (Reichegl, M., ed.) III Sect. E, pp. 335-368, CRC Press, Palm Beach, FL.
13. Denko, C.W. (1976) *Agents Actions 6,* 636-641.
14. Bonta, I.L., Parnham, M.J., and Adolfs, M.J.P. (1977) *Prostaglandins 14,* 295-307.
15. Gyllenhammar, H., Ringertz, B., Becker, W., Svensson, J., and Palmblad, J. (1986) *Immunol. Lett. 13,* 185-189.
16. Opmeer, F.A., Adolfs, M.J.P., and Bonta, I.L. (25) *J. Lipid Res. 25,* 262-268.
17. Stenson, W.F., Prescott, S.M., and Sprecher, H. (1984) *J. Biol. Chem. 259,* 11784-11789.
18. Becker, W., Bruce, Å., and Larsson, B. (1983) *Ann. Nutr. Metab. 27,* 415-424.
19. Holman, R.T. (1960) *J. Nutr. 70,* 405-410.
20. Salari, H., Braquet, P., Naccache, P., and Borgeat, P. (1985) *Inflammation 9,* 127-138.
21. Poubelle, P., Beaulieu, A.D., Nadeau, M., Laviolette, M., and Borgeat, P. (1986) in *Advances in Inflammation Res. 11* (Oterness, I., Caperola, R., and Wong, S., eds.), pp. 17-29, Raven Press, New York, NY.
22. Borgeat, P. (1984) *Prostaglandins 27,* 356-358.
23. Ringertz, B., Palmblad, J., Radmark, O., and Malmsten, C. (1983) *FEBS Lett. 147,* 180-182.
24. Ringertz, B., Palmblad, J., and Lindgren, J.-Å. (1985) *J. Clin. Lab. Med. 106,* 132-141.
25. Fletcher, M.P., Seligmann, B.E., and Gallin, J.I. (1982) *J. Immunol. 128,* 941-948.
26. Scatchard, G. (1949) *Ann. NY Acad. Sci. 51,* 660-672.
27. Seligmann, B., Chused, T.M., and Gallin, J.I. (1984) *J. Immunol. 133,* 2641-2646.
28. Bass, D.A., Olbrantz, P., Szejda, P., Seeds, M.C., and McCall, C.E. (1986) *J. Immunol. 136,* 860-866.
29. Kreisle, R.A., Parker, C.W., Griffin, G.L., Senior, R.M., and Stenson, W.F. (1985) *J. Immunol. 134,* 3356-3363.
30. Lohr, K.M., and Snyderman, R. (1982) *J. Immunol. 129,* 1594-1599.
31. Evans, J.F., Nathaniel, D.J., Zamboni, R.J., and Ford-Hutchinson, A.W. (1985) *J. Biol. Chem. 260,* 10966-10970.
32. Levine, L. (1983) *Biochem. Pharmacol. 32,* 3023-3026.
33. O'Flaherty, J.T., Thomas, M.J., Lees, C.J., and McCall, C.E. (1981) *Am. J. Pathol. 104,* 55-62.
34. Prescott, S.M., Zimmerman, G.A., and Seeger, A.R. (1984) *Biochem. Biophys. Res. Commun. 122,* 535-541.
35. Walsh, C.E., WAite, B.M., Thomas, M.J., and DeChatelet, L.R. (1981) *J. Biol. Chem. 256,* 7228-7234.
36. Ehrström, M., Harms-Ringdahl, M., and Alling, C. (1981) *Biochim. Biophys. Acta 644,* 175-182.
37. Gibson, R.A., Ncmurchie, E.J., Charnock, J.S., and Kneebone, G.M. (1984) *Lipids 19,* 942-951.
38. Stubbs, C.D., and Smith, A.D. (1984) *Biochim. Biophys. Acta 779,* 89-137.
39. Sha'afi, R.I., Molski, T.F.P., Borgeat, P., and Naccache, P.H. (1981) *Biochem. Biophys. Res. Commun. 103,* 766-773.
40. Palmblad, J., Wannemacher, R.W., Salem, N. Jr., Kuhns, D.B., and Wright, D.G., *J. Lab. Clin. Med.,* in press.
41. Holman, R.T., Johnson, S.B., Mercuri, O., Itarte, H.J., Rodrigo, M.A., and De Tomas, M.E. (1981) *Am. J. Clin. Nutr. 34,* 1534-1539.

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Synthesis of Platelet Activating Factor by Cholinephosphotransferase in Developing Fetal Rabbit Lung

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Developing fetal lung is a possible source of the platelet activating factor (PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) present in amniotic fluid of women in labor. We have assayed the microsomal activities of a specific enzyme for the de novo synthesis of PAF in developing fetal and neonatal rabbit lung, 1-alkyl-2-acetyl-glycerol-dependent dithiothreitol-insensitive cholinephosphotransferase. The specific activity of this enzyme increased from 0.92 to 3.60 nmol × min^{-1} × mg^{-1} protein between day 21 and day 31 of gestation. In contrast, during this same period the activity of the PAF-biosynthetic cholinephosphotransferase in developing rabbit kidney did not change significantly. The specific activity of the diacylglycerol-dependent, dithiothreitol-sensitive cholinephosphotransferase that catalyzes the final step in phosphatidylcholine biosynthesis was not altered during late gestation in either fetal lung or kidney. Previously, increased amounts of pulmonary PAF had been found during the latter stages of gestation (Hoffman, Truong and Johnston (1986) *Biochim. Biophys. Acta 879,* 88–96) and may be attributed to increased activity of the PAF biosynthetic enzymes found in this investigation. This elevated level of PAF in fetal lung may serve to facilitate breakdown of glycogen that provides, in part, the carbon and energy source for surfactant biosynthesis. In addition, PAF may be secreted in association with surfactant into amniotic fluid in which it may interact with amnion tissue and subsequently participate in the events associated with the initiation of parturition.
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The potent biologically active phospholipid, platelet activating factor (PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) first was identified with various pathological states such as inflammation, asthma and anaphylaxis (1,2). More recently, it is evident that PAF may have important normal cellular functions. We have proposed two potential physiological roles for PAF in reproductive biology: PAF may initiate glycogenolysis in fetal lung, the products of which provide energy and a carbon source for biosynthesis of the glycerophospholipids of surfactant (3–5), and PAF may be secreted from the fetal lung into amniotic fluid, in which it may interact with amnion to enhance the formation of prostaglandins by activation of the arachidonic acid cascade. Both prostanoids and PAF may facilitate the biochemical events of parturition (5,6).

PAF is only found in significant amounts in the amniotic fluid obtained from women in labor (7) and in part is associated with a lamellar body-enriched fraction containing surfactant. Since lamellar bodies are a primary secretory product of type II pneumonocytes of lung, we considered that fetal lung may be a potential source of the PAF present in amniotic fluid.

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Abbreviations: CPTase, cholinephosphotransferase; DTT, dithiothreitol; PAF, platelet activating factor; PC, phosphatidylcholine.

In previous investigations, we have reported a three-fold increase in the concentration of PAF in vivo in developing fetal rabbit lung during the latter stages of gestation (4) and also in rapidly differentiating human fetal lung explants cultured for six days (3). In both of these model systems, a three-fold increase was found in the activity of 2-lysoPAF:acetyl CoA acetyltransferase (3,4), a major regulatory enzyme of the remodeling pathway of PAF biosynthesis (8).

PAF also can be synthesized by 1-alkyl-2-acetyl-*sn*-glycerol:CDP-choline cholinephosphotransferase (CPTase) (9,10). This enzyme catalyzes the last step in the de novo pathway for PAF biosynthesis (11). In this investigation, we have examined the specific activity of this enzyme in developing fetal and neonatal rabbit lung and kidney.

MATERIALS AND METHODS

Chemicals. Cytidine 5′-diphospho[methyl-^{14}C]choline (56 μCi/μmol) and [^{3}H]acetyl CoA (500 mCi/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). Phospholipid standards were from Avanti Polar Lipids (Birmingham, AL). Fatty acid–free fraction V bovine serum albumin, 1-hexadecyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, and other general chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Unlabeled 2-lysoPAF was obtained from Calbiochem (La Jolla, CA).

Diradylglycerol preparation. 1-Alkyl-2-acetyl-*sn*-glycerol was synthesized as described by Woodard et al. (10). Briefly, 1-hexadecyl-*sn*-glycerol was chemically acetylated, the *sn*-3 acetate was cleaved by porcine pancreatic lipase treatment. The 1,2-isomer was separated from the 1,3-isomer on boric acid impregnated thin layer chromatography plates developed in chloroform:methanol (98:1.5, v/v). 1,2-Dioleoyl-*sn*-glycerol was purified chromatographically as above.

Tissue preparation. Timed pregnant New Zealand white rabbits (Hickory Hills, Flint, TX) were the source of fetal and adult tissues. Tissues were excised and rapidly homogenized (4 C) in 6:1 (vol/g tissue) with sucrose (0.25 M)/Tris-HCl (10 mM, pH 7.4)/EDTA (1 mM) using a Potter-Elvehjem vessel and a Teflon pestle. The suspension was centrifuged at 600 × g for 10 min to remove unbroken cells, cell debris and nuclei. The supernatant fraction was centrifuged at 18,000 × g for 15 min to obtain a pellet rich in mitochondria and lamellar bodies (12). The remaining supernatant fraction was centrifuged at 105,000 × g for 60 min to obtain the microsomal (pellet) and cytosolic fractions (supernatant). Protein concentrations were quantitated by the Bradford procedure (13).

Cholinephosphotransferase assay. The PAF biosynthetic activity of dithiothreitol (DTT)-insensitive CPTase (EC 2.7.8.16) was determined employing Tris-HCl (100 mM, pH 8.0 at 37 C), DTT (10 mM), EGTA (0.5 mM), 0.1% bovine serum albumin, $MgCl_2$ (10 mM), 1-hexadecyl-2-acetyl-*sn*-glycerol (0.2 mM in ethanol, final

ethanol concentration equals 2.5%), 25 μg microsomal protein and CDP-[methyl-^{14}C]choline (100 μM, 1 μCi/mmol) according to the procedure of Renooij and Snyder (9). Assay of phosphatidylcholine (PC) biosynthetic activity of CPTase (EC 2.7.8.2) was as above except the reaction was at pH 8.5, contained no DTT, and utilized 1,2-dioleoyl-*sn*-glycerol (0.2 mM) as substrate. The incubation was for 10 min at 37 C. PAF and PC were separated by thin layer chromatography on Kieselgel 60 plates (E. Merck, Darmstadt) developed in chloroform:methanol: concentrated ammonium hydroxide (60:35:8, v/v/v). Radioactivity comigrating with authentic PAF and PC added as carrier (20 μg each) was determined, and the values for both CPTase assays were corrected for the incorporation of [^{14}C]choline in the absence of diradylglycerol substrate. The assay was linear to 75 μg microsomal protein and for 20 min.

LysoPAF:acetyl-CoA acetyltransferase assay. The activity of this enzyme was determined using a modification of the procedure described by Wykle et al (8). Briefly, the reaction mixture includes Tris-HCl (30 mM, pH 7.4 at 37 C), DTT (1 mM), $CaCl_2$ (10 μM), 2-lysoPAF (40 μM), 0.025% bovine serum albumin, [^{3}H]acetyl CoA (250 μM, 6.4 μCi/μmol), 50 μg microsomal protein, and was incubated for 15 min at 37 C. PAF was separated and radioactivity determined as described. The assay was linear to 120 μg microsomal protein and for 20 min.

RESULTS

The PAF biosynthetic CPTase reaction is distinguished from the PC biosynthetic activity primarily by the inhibitory action of the sulfhydryl agent, dithiothreitol, on the latter activity (9). As illustrated in Table 1, when PAF biosynthesis was assayed utilizing 1-alkyl-2-acetyl-*sn*-glycerol as substrate the addition of DTT (10 mM) only slightly stimulated (19%) its biosynthesis. In contrast, PC biosynthesis with the same fetal rabbit lung microsomal preparation is inhibited by 95% in the presence of DTT. When alkyl-acetyl-glycerol was the substrate for the CPTase reaction, negligible amounts of [^{14}C]choline from CDP-[^{14}C]choline were incorporated into PC. Similarly, when dioleoyl-glycerol was utilized as substrate, [^{14}C] incorporation into PAF was less than 2% of that incorporated into PC.

The PAF- and PC-biosynthetic CPTase activities primarily were (66–68%) localized in the 105,000 × *g* (microsomal) pellet of adult rabbit lung (Table 2). This subcellular distribution of the DTT-insensitive CPTase is similar to that reported in rat liver and spleen (9). The distribution of CPTase activity nearly was identical to that of acetyltransferase in rabbit lung (Table 2).

Substrate specificity characteristics of the PAF and PC biosynthetic reactions were investigated in adult rabbit lung microsomal preparations. Reaction mixtures at pH 8.0 containing DTT favor PAF biosynthesis, however, inclusion of dioleoyl-glycerol at an equimolar concentration as the alkyl-acetyl-glycerol substrate (0.2 mM) causes a 36% decrease in PAF biosynthesis when compared to that

TABLE 1

Dithiothreitol Sensitivity of PAF and PC Biosynthetic Cholinephosphotransferases in Fetal Rabbit Lung Microsomes

Substrate	Specific activity (nmol × min^{-1} × mg^{-1} protein)	
	−DTT	+10 mM DTT
Alkyl-acetyl-glycerol	5.48 ± 1.08	6.54 ± 0.67
Dioleoyl-glycerol	1.49 ± 0.18	0.07 ± 0.04

Values (±S.E.M.) are from duplicate analysis in at least three separate samples.

TABLE 2

Subcellular Distribution of DTT-insensitive and DTT-sensitive Cholinephosphotransferases (CPTase) and LysoPAF:Acetyl-CoA Acetyltransferase Specific Activities in Adult Rabbit Lung

Enzyme	Subcellular fraction			
	Supernatant (600 × *g*)	Pellet (18,000 × *g*)	Pellet (105,000 × *g*)	Supernatant (105,000 × *g*)
	nmol × min^{-1} × mg^{-1} protein			
DTT-insensitive PAF-CPTase	1.06 ± 0.03	2.82 ± 0.07 (33)	5.83 ± 0.43 (67)	0
DTT-sensitive PC-CPTase	0.77 ± 0.13	1.07 ± 0.06 (34)	2.10 ± 0.07 (66)	0.01 ± 0
Acetyltransferase	0.54 ± 0.10	0.73 ± 0.11 (28)	1.78 ± 0.30 (69)	0.07 ± 0.01 (3)

Values are means (±S.E.M.) from four (CPTase) or three (acetyltransferase) separate experiments.

Values in parentheses are percent of total activity present in each subcellular fraction. Average recovery of the PAF-CPTase, PC-CPTase and acetyltransferase activities were 119, 63 and 98%, respectively.

containing only alkyl-acetyl-glycerol (i.e., 4.07 vs 6.33 nmol × min^{-1} × mg^{-1} protein, Table 3). Similarly, in a corresponding experiment alkyl-acetyl-glycerol inhibited PC biosynthesis by 36% (2.72 to 1.74 nmol × min^{-1} × mg^{-1} protein) in incubation mixtures at pH 8.5 and from which DTT was omitted. In the presence of equimolar concentrations of both substrates, total product formation, (2.13 nmol PAF formed × min^{-1} × mg^{-1} protein plus 1.74 nmol PC formed × min^{-1} × mg^{-1} protein) approaches a rate observed using only alkyl-acetyl-glycerol as substrate (i.e., 4.05 nmol PAF formed × min^{-1} × mg^{-1} protein). Although these results would be consistent with the view that both diradylglycerols are substrates for a single enzyme, the precise mechanism must await purification and characterization of the enzymes involved.

During late gestation in the rabbit (term = 31–32 days), only the activity of the PAF biosynthetic (DTT-insensitive) CPTase of fetal lung increased significantly (Figure 1). In contrast, this enzymatic activity was not altered significantly in fetal kidney. During neonatal development, the PAF biosynthetic activity increased in both lung and kidney to reach the adult level. The PC

TABLE 3

Cholinephosphotransferase Diradylglycerol Substrate Specificity in Adult Rabbit Lung Microsomes

Substrate added (200 μM)		Alkyl-acetyl glycerol	Dioleoyl glycerol	Both
Reaction conditions	Product assayed	nmol × min^{-1} × mg^{-1} protein		
+DTT pH 8.0	PAF	6.33 ± 0.54	0.02 ± 0.01	4.07 ± 0.52
	PC	0.03 ± 0.03	0.02 ± 0.01	0
−DTT pH 8.5	PAF	4.05 ± 0.08	0.09 ± 0.04	2.13 ± 0.35
	PC	0	2.72 ± 0.93	1.74 ± 0.91

Values are the means (± SEM) of three separate experiments.

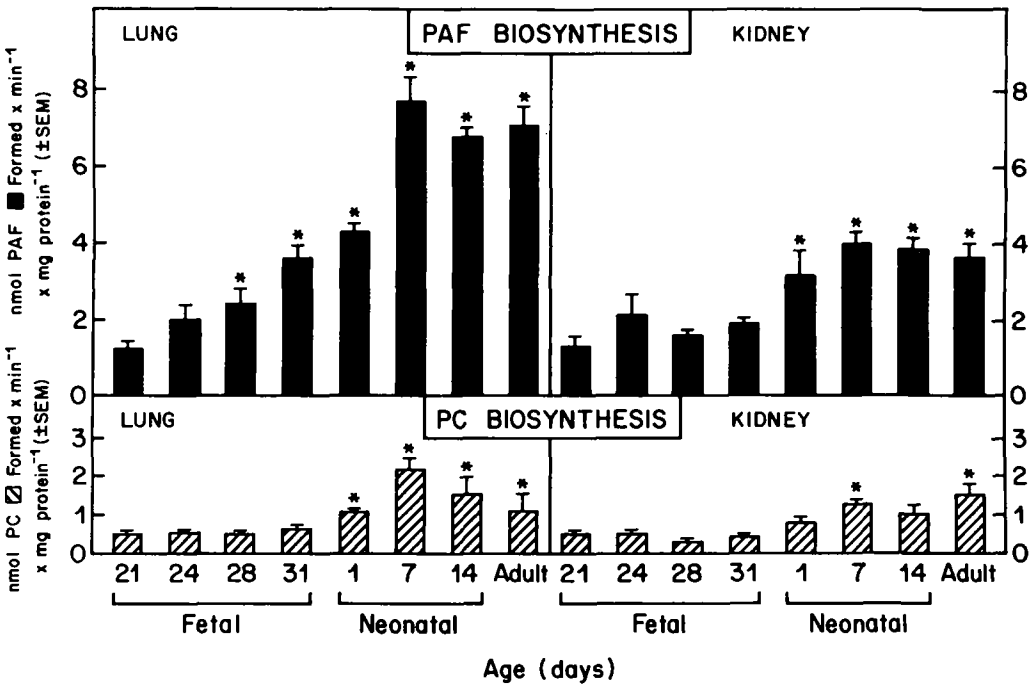

FIG. 1. Specific activity of the PAF (DTT-insensitive) and PC (DTT-sensitive) biosynthetic cholinephosphotransferases in microsomes of fetal, neonatal and adult rabbit lung and kidney. Biosynthetic activities were determined as described in Materials and Methods. Bars represent the mean ± SEM for a minimum of three separate experiments. * Indicates a significant difference ($p > 0.01$) from the day 21 value as determined by Student's t-test.

biosynthetic (DTT-sensitive) activity of CPTase was not altered during gestational development in the lung or kidney. In both neonatal lung and kidney, the PC-biosynthetic CPTase was elevated over the fetal level. In kidney, an adult level of enzymatic activity was reached and maintained; however, in neonatal lung, a peak of activity is reached at seven days followed by a return to an adult level comparable to that in the fetal lung.

We previously had found (4) that the microsomal 2-lysoPAF:acetyl CoA acetyltransferase specific activity in fetal rabbit lung microsomes increased significantly between days 21 and 24 of gestation (from 116 to 392 pmol PAF formed $\times$ min^{-1} $\times$ mg^{-1} protein) and remained elevated through day 31. The acetyltransferase activity in adult rabbit lung microsomes was further increased to 1780 $\pm$ 300 (S.E.M.) pmol $\times$ min^{-1} $\times$ mg^{-1} protein. In kidney (4), the enzyme activity also increased during the final 10 days of fetal development (from 68 to 187 pmol $\times$ min^{-1} $\times$ mg^{-1} protein). The adult rabbit kidney acetyltransferase (305 $\pm$ 50 [S.E.M.] pmol $\times$ min^{-1} $\times$ mg^{-1} protein) was elevated over fetal activities; however, neither the fetal nor adult rabbit kidney acetyltransferase activities reach the level of that in the lung at the corresponding age of development.

DISCUSSION

The insensitivity of the PAF biosynthetic activity of CPTase to DTT (Table 1) and localization in the 105,000 $\times$ *g* pellet (microsomal) fraction in rabbit lung (Table 2) are indicative of the presence of a similar enzymatic activity in this tissue to that described in adult rat kidney (9). The PC biosynthetic activity of CPTase in lung markedly was inhibited by DTT (Table 1) and each substrate, alkyl-acetyl-glycerol and diacyl-glycerol, influenced the ability of CPTase to utilize the other substrate (Table 3). The PAF and PC biosynthetic activities of cholinephosphotransferase(s) are thought to be two distinct enzymes (10). However, a distinction between the presence of one or two enzymes in lung tissue must await purification of the enzyme and further evaluation of the physical properties of the substrates utilized.

In this investigation, a stepwise increase in the activity of alkyl-acetyl-glycerol-dependent PAF-biosynthetic CPTase was found in fetal rabbit lung microsomes. This developmental pattern of the de novo PAF biosynthetic pathway is consistent with that found previously for a major regulatory enzyme of the remodeling pathway, lysoPAF:acetyl CoA acetyltransferase (4). However, the specific activity of the PAF biosynthetic CPTase (Figure 1) was found to be approximately 10-fold higher than the acetyltransferase activity in fetal lung and kidney and thus may contribute more significantly to the total pool of PAF in these tissues. A similar relationship between the activities of these two enzymes has been reported in adult rat kidney (10). Lipid substrate availability, however, may limit the in vivo activity of the PAF-biosynthetic CPTase as this substrate, alkyl-acetyl-glycerol, is derived from numerous enzymatic steps of the de novo ether lipid pathway. In contrast, we have quantitated the acetyltransferase substrate, lysoPAF, in the developing fetal lung and found it in great excess as compared with PAF concentrations (3,4).

The PC biosynthetic specific activity of CPTase did not change significantly between day 21 and day 31 in fetal rabbit lung (Figure 1) and confirms our earlier observations (14) of the ontogenic development of this enzymatic activity. Although it originally was suggested that CPTase was one of the primary enzymes involved in regulation of surfactant PC biosynthesis during fetal development (15,16), in rabbit it appears not to be the case (14,17–19). The PAF biosynthetic specific activity of CPTase was three- to six-fold higher than the PC biosynthetic activity throughout fetal lung maturation (Figure 1). This same relationship of enzymatic activity was observed in adult rat kidney (10) and may reflect a preferential synthesis of PAF under certain physiological circumstances. However, this paradox of higher PAF biosynthetic capacity compared to PC biosynthesis also may be attributable to the substrate availability in the developing fetal lung.

The activities of the PAF biosynthetic CPTase (Figure 1) and acetyltransferase (4) both increase at a time (starting at day 24 of gestation) when the epithelial lining of fetal rabbit lung rapidly is differentiating into type II pneumonocytes (20). It is well-established from both morphological and biochemical studies that during differentiation of type II pneumonocytes, the concentration of glycogen rapidly decreases (21–23) in association with increased synthesis of the glycerophospholipids unique to the surfactant fraction of these cells (23–26). We have demonstrated that increased concentrations of PAF also are found in fetal lung during this time period (3,4), possibly reflecting the increase in the activities of CPTase and acetyltransferase. We have proposed (3–5) and recently obtained experimental evidence (Hoffman and Johnston, unpublished data) that PAF may stimulate glycogenolysis in fetal lung in a manner similar to that originally reported by Shukla et al. (27) in isolated, perfused rat liver. Thus, PAF initiates or potentiates the breakdown of glycogen that may supply in part the energy and carbon moieties necessary for synthesis of surfactant glycerophospholipids. Other investigators have suggested that this is the case in fetal lung during the latter stages of gestation (24,25) because it receives only a small fraction of the cardiac output and primarily will utilize glycogen for surfactant biosynthesis rather than circulating blood glucose (25,28). The increased amounts of PAF in fetal lung also may become associated with surfactant and be secreted out into the amniotic fluid during late gestation just prior to parturition. This increased level of PAF in amniotic fluid is found in women at term and in labor (7). Thus, the presence of PAF in amniotic fluid only at the time of parturition is indicative of its role in the initiation of this process (6,29). The PAF that is present in amniotic fluid may interact with amnion tissue to stimulate prostaglandin synthesis (30), which in turn may participate in the events of parturition. PAF also directly can elicit the contraction of rat (31) and human (32) myometrium. Thus, it appears that the PAF synthesized in fetal lung, the last major fetal organ system to develop, also may be involved in the events of labor.

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REFERENCES

1. Hanahan, D.J. (1986) *Annu. Rev. Biochem. 55,* 483-509.
2. Lee, T-c., and Snyder, F. (1985) in *Phospholipids and Cellular Regulation* (Kuo, J.F., ed.) Vol. 2, pp. 1-39, CRC Press, Boca Raton, FL.
3. Hoffman, D.R., Truong, C.T., and Johnston, J.M. (1986) *Am. J. Obstet. Gynecol. 155,* 70-75.
4. Hoffman, D.R., Truong, C.T., and Johnston, J.M. (1986) *Biochim. Biophys. Acta 879,* 88-96.
5. Hoffman, D.R., and Johnston, J.M. (1987) in *New Horizons in Platelet Activating Factor Research* (Winslow, C.M., and Lee, M.L., eds.) pp. 51-55, John Wiley and Son, New York, NY.
6. Johnston, J.M., Bleasdale, J.E., and Hoffman, D.R. (1987) in *Platelet-Activating Factor and Related Lipid Mediators* (Snyder, F., ed.) pp. 375-402, Plennum Press, New York, NY.
7. Billah, M.M., and Johnston, J.M. (1983) *Biochem. Biophys. Res. Commun. 113,* 51-58.
8. Wykle, R.L., Malone, B., and Snyder, F. (1980) *J. Biol. Chem. 255,* 10256-10260.
9. Renooij, W., and Snyder, F. (1981) *Biochim. Biophys. Acta 663,* 545-556.
10. Woodard, D.S., Lee, T-c., and Snyder, F. (1987) *J. Biol. Chem. 262,* 2520-2527.
11. Lee, T-c., Malone, B., and Snyder, F. (1986) *J. Biol. Chem. 261,* 5373-5377.
12. Okazaki, T., Johnston, J.M., and Snyder, J.M. (1982) *Biochim. Biophys. Acta 712,* 283-291.
13. Bradford, M.M. (1976) *Anal. Biochem. 72,* 248-254.
14. Schultz, F.M., Jimenez, J.M., MacDonald, P.C., and Johnston, J.M. (1974) *Gynecol. Invest. 5,* 222-229.
15. Farrell, P.M., Lundgren, D.W., and Adams, A.J. (1974) *Biochem. Biophys. Res. Commun. 57,* 696-701.
16. Oldenborg, V., and van Golde, L.M.G. (1976) *Biochim. Biophys. Acta 441,* 433-442.
17. Tsao, F.H.C., and Zachman, R.D. (1977) *Pediatr. Res. 11,* 858-861.
18. Heath, M.F., and Jacobson, W. (1984) *Pediatr. Res. 18,* 395-401.
19. Gluck, L., Sribney, M., and Kulovich, M.V. (1967) *Pediatr. Res. 1,* 247-265.
20. Snyder, J.M., Mendelson, C.R., and Johnston, J.M. (1985) in *Pulmonary Development: Transition from Intrauterine to Extrauterine Life* (Nelson, G.H., ed.), pp. 19-46, Marcel Dekker, New York, NY.
21. Kikkawa, Y. (1975) in *Pulmonary Physiology of the Fetus, Newborn and Child* (Scarpelli, E., ed.), pp. 37-60, Lea and Febiger, Philadelphia, PA.
22. Shelley, H.J. (1961) *Br. Med. Bull. 17,* 137-143.
23. Snyder, J.M., Johnston, J.M., and Mendelson, C.R. (1981) *Cell Tissue Res. 220,* 17-25.
24. Maniscalco, W.M., Wilson, C.M., Gross, I., Gobran, L., Rooney, S.A., and Warshaw, J.B. (1978) *Biochim. Biophys. Acta 530,* 333-346.
25. Bourbon, J.R., Rieutort, M., Engle, M.J., and Farrell, P.M. (1982) *Biochim. Biophys. Acta 712,* 382-389.
26. van Golde, L.M.G. (1976) *Am. Rev. Respir. Dis. 114,* 977-1000.
27. Shukla, S.D., Buxton, D.B., Olson, M.S., and Hanahan, D.J. (1983) *J. Biol. Chem. 258,* 10212-10214.
28. Maniscalco, W.M., Finkelstein, J.N., and Parkhurst, A.B. (1982) *Biochim. Biophys. Acta 11,* 49-58.
29. Bleasdale, J.E., and Johnston, J.M. (1984) *Rev. Perinatal Med. 5,* 151-191.
30. Billah, M.M., Di Renzo, G.C., Ban, C., Truong, C.T., Hoffman, D.R., Anceschi, M.M., Bleasdale, J.E., and Johnston, J.M. (1985) *Prostaglandins 30,* 841-850.
31. Nishihira, J., Ishibashi, T., Imai, Y., and Muramatsu, T. (1984) *Lipids 19,* 907-910.
32. Tetta, C., Montrucchio, G., Alloatti, G., Roffinello, C., Emanuelli, G., Benedetto, C., Camussi, G., and Massobrio, M. (1986) *Proc. Soc. Exptl. Biol. Med. 183,* 376-381.

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Uptake of Artificial Model Remnant Lipoprotein Emulsions by the Perfused Rat Liver[1]

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In comparison with their precursor lipoproteins, the remnants of the triacylglycerol-rich lipoproteins are reduced in contents of triacylglycerols and apolipoproteins AI and AIV, whereas the contents of cholesterol (free and esterified) and apolipoprotein E are increased. In this study, lipid emulsion models of remnant lipoproteins were used to explore which of these factors are necessary for physiological rates of remnant uptake by the perfused rat liver. Uptake rates of lipid emulsion models of remnant lipoproteins in the presence of apolipoprotein E were similar to in vivo uptake rates.

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After entering the bloodstream, the triacylglycerol-rich lipoproteins are hydrolyzed by lipoprotein lipase. The residual core remnant lipoprotein particles subsequently are taken up mostly by the liver (1,2).

The perfused rat liver was used to show that the uptake of remnants was more rapid than the uptake of the undegraded precursor triacylglycerol-rich lipoproteins (3–11). Uptake of remnants into hepatic parenchymal cells was by receptor-mediated endocytosis, with apolipoprotein E as the ligand (12,13), while the plasma C apolipoproteins were inhibitory. It was suggested that the different ratios of the E and C apolipoproteins between triacylglycerol-rich lipoproteins and remnants explained the differences in hepatic uptake (14–17). The uptake of nonremnant-like triacylglycerol emulsions by perfused livers was promoted by apolipoprotein E and impeded by the C apolipoproteins (18,19).

Recent studies in our laboratory showed that triacylglycerol-phospholipid emulsions containing small amounts of cholesterol were metabolized like natural chylomicrons (20). Increased amounts of cholesterol changed the metabolism of triacylglycerol-phospholipid emulsions so that they behaved like remnants with rapid uptake by the liver but without significant lipolysis (21). We found that this change in metabolism corresponded with changes in the amounts of apolipoproteins E and C that associated with the emulsions after incubation with whole plasma or with plasma subfractions.

We now report the uptake of the remnant-like emulsions by perfused rat livers. Our findings show that remnant-like emulsion models of lipoproteins are capable of imitating the metabolism of natural remnants only when sufficient apolipoprotein E is present.

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Abbreviation: EDTA, ethylenediamine tetraacetic acid.

METHODS

Materials. Male rats of the Holtzman strain were purchased from Charles River Breeding Laboratories (Wilmington, MA). Triolein, cholesteryl oleate and cholesterol were from Nu-Chek Prep (Elysian, MN), and egg lecithin was from Lipid Products (Surrey, UK). All had class purity >99% by thin layer chromatography. Bovine serum albumin (Fraction V, fatty acid poor, Miles Biochemicals, Kanakee, IL) before use was dialyzed for 48 hr as a 16% solution against six changes of Krebs-Ringer bicarbonate buffer in an artificial kidney dialysis machine. The dialyzed albumin solution then was stirred at 4 C for 16 hr with the emulsion particles from Intralipid (Cutter Laboratories, Berkeley, CA), previously separated from the excess phospholipid liposomes that are present in Intralipid by centrifugal flotation at 27,000 rpm for one hr at 4 C in the Beckman SW28 rotor. The ratio of Intralipid triacylglycerols to albumin solution was 25 g/l, and all treatments were under an atmosphere of 95% oxygen and 5% carbon dioxide to maintain physiological concentrations of calcium ion in the albumin-buffer solution. Finally, the emulsion particles were removed from the albumin solution by centrifugation under the same conditions. This treatment was required to purify the albumin of material that otherwise was inhibitory for physiological uptake by the perfused livers.

Preparation of emulsions. Emulsions of the required compositions were prepared by sonication and purified by ultracentrifugation as recently described (20,21). Mixtures of lipids in solvents were dispensed into vials, and tracer amounts of radioactive [^{3}H]cholesteryl oleyl ether (22) were added. Solvents were removed by evaporation under a stream of nitrogen followed by vacuum desiccation overnight. The mixtures (total mass 100 mg) were emulsified in 8 ml of 2.785 molal NaCl solution by sonication with a 1 cm probe in a Branson Cell Disruptor (Danbury, CT) at ~55 C. The mixture then was centrifuged using density gradients previously described (23) to obtain emulsion particles sized 50–100 nm in diameter. In brief, the crude sonicated emulsion was placed under gradients made from 1.065–1.006 g/ml density NaCl solutions, then larger emulsion particles were first removed by centrifugation in the Beckman SW41 rotor at 20 C for 22 min at 20,000 rpm. After removal of the large particles floating at the surface, the gradient was then recentrifuged at 20 C for 20 min at 40,000 rpm to float the desired particles to the top of the gradient. Smaller particles and liposome material remained in deeper regions of the gradient.

Preparation of chylomicron remnants. Mesenteric lymph was obtained by cannulation of the cisterna chyli of 250 g male rats. After surgery, the rats were placed in individual cages and fed regular rat chow. Tap water was available freely, and 0.15 M NaCl solution was delivered at 2.0 ml/hr through a gastric tube inserted at

the time of surgery. Lymph flow was 2.8 ± 0.81 ml/hr (mean±SD, n=16). To obtain radioactive chylomicrons on the second day after surgery, a meal with 75 μCi [1,2(n)-^{3}H]cholesterol (Amersham Corp., Arlington Heights, IL) dissolved in 0.5 ml of triolein was injected through the gastric tube.

The milky lymph was collected from one-half to six hr after the dose into vessels containing ethylenediamine tetraacetic acid (EDTA), gentamycin and reduced glutathione to final concentrations of 2.7 mM, 0.1 mg/ml and 1.6 mM, respectively. Collections were at room temperature (18-25 C), and the lymph was not cooled at any time. Cells were removed by centrifugation at 1,500 rpm for 15 min, then solid KBr 0.14 g/ml was added to bring the density to 1.10 g/ml. After degassing in vacuo, 14 ml of the lymph was placed under 24 ml continuous linear density gradients 1.006-1.10 g/ml for centrifugation in the Beckman SW28 rotor. Chylomicrons were obtained from the top 0.5 cm of the gradient after centrifugation at 20 C and 27,000 rpm for 4.0×10^{10} radian^2/sec, plus deceleration without braking. As previously described (16), the chylomicrons then were injected via the tail vein into 24 hr-fasted, functionally hepatectomized rats and allowed to circulate for 30 min before exsanguination by cardiac puncture. The remnants then were separated from the plasma by centrifugation at 39,000 rpm in the SW41 rotor at 20 C for 20 hr in the density gradient described above.

Apolipoproteins. Rat and human apolipoproteins were prepared from plasma very low-density lipoproteins and high-density lipoproteins by sequential centrifugations (24) in the Beckman 70Ti rotor. The lipoproteins were dialyzed against 0.01% EDTA, pH 8.0, lyophilized, then delipidated at 6 C with graded mixtures of ethanol/diethyl ether and finally washed with pure peroxide-free ether (25). The apolipoproteins were fractionated by gel chromatography on a 90 × 2.5 cm Sephacryl (Pharmacia, Piscataway, NJ) S-300 column equilibrated and eluted with 3 M guanidine HCl, 0.1 M Tris HCl (pH 8.0), 0.27 mM EDTA, 3.1 mM NaN_3 and 0.01 mM dithiothreitol (26). Human delipidated very low-density lipoproteins were taken up in buffer, centrifuged to sediment insoluble apolipoprotein B, then applied to the column to separate the E and C apolipoproteins.

To separate the apolipoproteins from human high-density lipoproteins, two columns were necessary, first with dithiothreitol omitted to obtain apolipoprotein AI and apolipoprotein AII (dimer) in one peak, then the C apolipoproteins as a group. After dialysis and lyophilization, the AI/AII mixture was chromatographed in the presence of dithiothreitol to separate AI and AII as the monomer.

The delipidated rat high-density lipoproteins were chromatographed as above to obtain the C apolipoproteins but apolipoproteins AIV, E and AI were distributed in an asymmetric peak that was divided into two pools containing either AIV and E, or E and AI. Each pool was then reapplied to a heparin-Sepharose affinity column (Pharmacia) as described by Shelburne and Quarfordt (27). Then, apolipoprotein AI or AIV were eluted with buffer containing 0.15 M NaCl before apolipoprotein E was eluted with buffer containing 2 M NaCl. Finally, all apolipoproteins were dialyzed against 0.01% EDTA pH 8.0 and lyophilized. Purity was monitored by polyacrylamide gel electrophoresis. Apolipoproteins were incubated with the emulsions in the proportions of 100 μg/mg lipid for two to three hr before adding to the perfusate.

Liver perfusions. Livers from overnight-fasted Holtzman rats weighing 244 ± 34 g were perfused in a recirculating system as described previously (28,29). Perfusate volume was ~150 ml of 4% albumin and 15 mM glucose in Krebs Ringer buffer, (pH 7.4) at 37 C. Bile was diverted by cannulation of the common bile duct. Sodium taurocholate continuously was infused into the system at 36 μmol/hr (30), and the pH was maintained with a pH-stat delivering 0.3 M NaOH.

The livers were equilibrated for 15 min in the system before a labeled emulsion or lipoprotein suspension was added to the perfusate reservoir, after removing the 8 μm Millipore filter that initially was present in the circuit. The perfusion rate was 59 ± 6.1 ml/min. Samples of 1.0 ml were taken at intervals of 4, 6, 8, 10, 12, 14, 17 and 20 min for measurement of residual radioactivity in the medium.

RESULTS

Prepared as described, the remnant-like emulsions had the composition triacylglycerols 73%, cholesteryl esters 5%, cholesterol 10% and phospholipids 12%. Figure 1

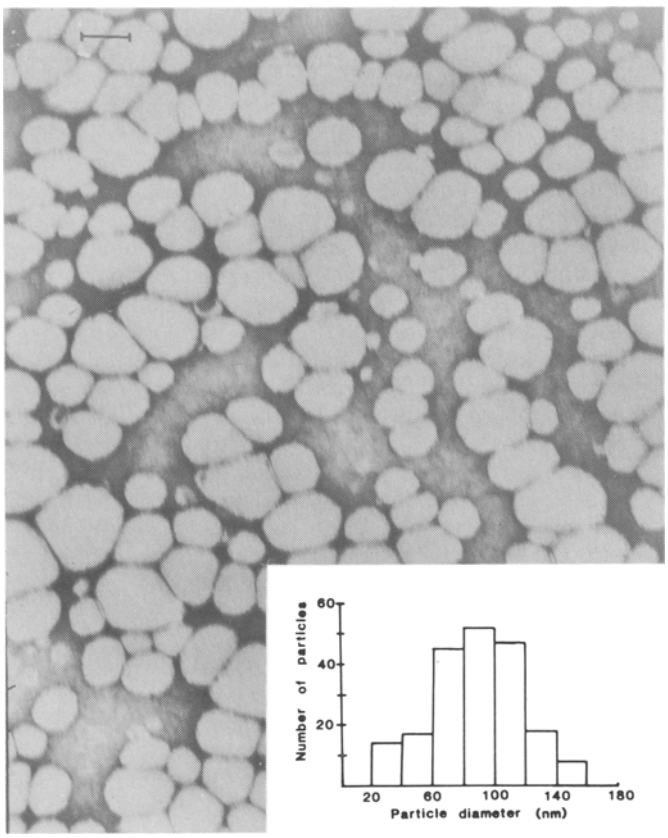

FIG. 1. Electron microscope appearance of the emulsions stained with 2% phosphotungstate. The bar indicates 100 nm. Some of the particles appear nonspherical, perhaps because of distortion from crowding on the grid. Particle diameters were measured as the means of two perpendicular measurements using a graticule, and the distribution shown is plotted as a histogram.

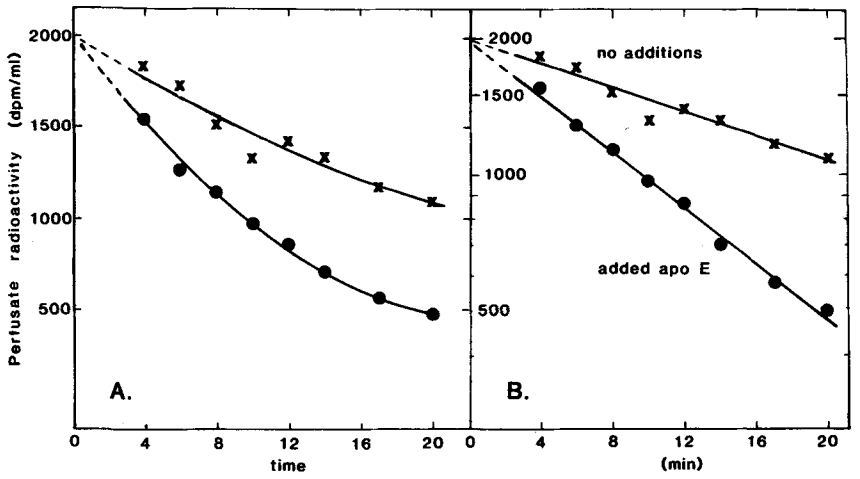

FIG. 2. The pattern of removal from the perfusate of the added emulsion radioactivity, plotted on linear and log-linear coordinates (A and B). Uptake was exponential. Added apolipoprotein E increased the rate of uptake. Uptake of natural remnants was almost identical with the emulsion in the presence of apolipoprotein E.

TABLE 1

Effect of Conditioning of the Perfusion Medium with Intralipid on the Removal of the Remnant-like Emulsion[a]

Perfusate	Rate of removal (%/min)
Unconditioned	0.1, 0.3
Conditioned	2.4 ± 0.5 (5)

[a]The removal rates were derived from the slopes of the exponential curves fitted to the data of radioactivity remaining in the perfusate collected during the first 20 min after adding the emulsions. Results are mean ± SD, (n)=number of experiments or each of duplicate experiments. The albumin used in the perfusion medium was treated as described in the Methods section. The mass of added emulsion was ≅2 mg total lipid.

TABLE 2

Effects of Apolipoproteins on Uptake of Remnant-like Emulsions[a]

Added apolipoprotein	Rate of removal (%/min)
None	2.4 ± 0.5 (5)
E (rat or human)	5.8 ± 1.7[b] (6)
C's (rat or human)	2.2 ± 1.1 (5)
AI (rat)	2.0 ± 0.5 (5)
AII (human)	1.1, 1.3
AIV (rat)	1.1

[a]Apolipoproteins were added to the emulsions in the proportion of 100 μg/mg lipid two to three hr before the liver perfusion. Where either rat or human apolipoproteins were used, uptake rates were similar with both. Other experimental details are as in Table 1.

[b]Significantly faster than with no addition, $p < 0.01$.

shows the electron microscope appearance of the emulsion after negative staining, and the histogram of particle sizes shows that most were between 60 and 120 nm in diameter. Although the emulsions were stable for at least one week, they always were used for perfusion studies within three days of preparation.

The livers continued to produce bile through the period of perfusion. Average bile flow was 0.265 ± 0.15 ml during the perfusion period (n=26). Not more than 0.1% of added emulsion or lipoprotein radioactivity was recovered in the bile. Figure 2 shows the pattern of removal of added radioactivity from the perfusate. Removal rates were exponential.

The requirement for preconditioning of the albumin used in the perfusion medium by treatment with Intralipid emulsion particles is shown by the data of Table 1. Without conditioning, the rate of removal of the added labeled emulsion from the perfusion system was very slow. Conditioning with Intralipid increased the rate by up to 20-fold and also increased the amount of radioactivity recovered in the liver (data not shown). Also effective in accelerating the rate of removal was triacylglycerol-rich emulsion added to the unconditioned medium during the perfusion with the radioactive test emulsion.

The effects of added apolipoproteins on uptake of the remnant-like emulsion are shown in Table 2. The extent of association of apolipoproteins with high-cholesterol emulsion was reported (21). Addition of apolipoprotein E accelerated the rate of removal, whereas apolipoproteins C or AI had little effect. Apolipoproteins AII and AIV appeared to be inhibitory for uptake but the numbers of experiments were insufficient for firm conclusions to be drawn.

Apolipoprotein E accelerated the rate of removal of chylomicrons from the perfusate as shown in Table 3 so that the removal was similar to that of natural remnants. Note that with added apolipoprotein E, the removal rate of the remnant-like emulsion (Table 2) also was similar to that of the natural remnants. With natural remnants, added apolipoproteins AI and AII appeared to slow the removal rate but again small numbers of observations prevent firm conclusions.

TABLE 3

Effects of Apolipoproteins on Uptake of Chylomicrons and Remnants[a]

Added apolipoprotein	Rate of removal (%/min)	
	Chylomicrons	Remnants
None	3.4	6.7 ± 2.3 (3)
E (rat)	6.5	
AI (rat)		4.3
AIV (rat)		4.4

[a]Additions of apolipoproteins and other experimental details as described in Tables 1 and 2.

DISCUSSION

The ligand for uptake of chylomicron remnants by the liver is apolipoprotein E (10), and hepatic receptors mediating remnant binding and uptake are designated B,E and E receptors (13,31). Because Watanabe heritable hyperlipemic rabbits that lack B,E receptors are able to metabolize chylomicron remnants efficiently (32), it must be concluded that the E receptors are implicated in remnant clearance. Similarly, chylomicrons appear to be metabolized normally in humans homozygous for defects in the B,E receptor.

We recently described the metabolism of emulsions prepared to model the in vivo metabolism of chylomicrons and remnants (20,21). Triacylglycerol-rich emulsions with low cholesterol content were metabolized like chylomicrons, whereas when the content of free cholesterol was high, the metabolism was like remnants. The emulsions modeling remnants were not hydrolyzed by lipoprotein lipase in peripheral vascular beds but were taken up by the liver without extensive depletion of triacylglycerols.

The differences in metabolism between emulsions with low or high cholesterol contents probably were explained by differences in affinity for the plasma apolipoproteins that associated with the emulsions after their intravenous injection. In vitro bindings of apolipoproteins AI and C were decreased by 35% and 26% for the emulsions with high cholesterol content, whereas the binding of apolipoprotein E was increased by 9% (21).

Compared with their precursor chylomicrons, remnants are high in their content of free cholesterol (1,16) so the same changes in lipoprotein affinities can be expected and have been observed (14,16). Thus, the increase in cholesterol content in remnants serves to facilitate the association with apolipoprotein E, which then mediates binding to the hepatic receptors.

In the present experiments with added apolipoprotein E, emulsions high in cholesterol were taken up by the liver similarly to natural chylomicron remnants, as shown by the data of Tables 2 and 3. Therefore, emulsions with high cholesterol content and with bound apolipoprotein E are adequate as models of chylomicron remnants, at least in terms of their uptake by the perfused liver.

In other studies of hepatic uptake of triacylglycerol-rich emulsions, the contents of cholesterol have been low, and therefore not a good model for remnants. Consistent with increased binding of the C apolipoproteins to emulsions low in cholesterol are the inhibitory effects on hepatic uptake produced by added C apolipoproteins. However, because emulsions high in cholesterol bind less of the C apolipoproteins inhibitory effects should be less. This expectation was supported by the data of Table 2.

Improvement of the rate of uptake by conditioning of the perfusion medium with Intralipid lipid particles probably also was explained by binding of apolipoproteins to the Intralipid emulsion. Commercial albumin was reported to contain contaminating apolipoprotein AI (33, 34). Our data provide preliminary evidence that apolipoproteins AII and AIV may be inhibitory for uptake, sufficient to justify further experiments specifically designed to test their possible role in remnant metabolism.

Recent in vivo data have shown clearance rates for chylomicron remnants and for emulsion models of remnants to be ≅10%/min for rats with a plasma volumes of ≅10 ml, and hepatic blood plasma flow of ≅6 ml/min. For a perfusate volume of ≅150 ml and a hepatic perfusion rate of ≅60 ml/min, as in our experiments, an uptake rate of ≅6.6%/min would be the expected physiological rate in the perfusion apparatus. Tables 2 and 3 show that for natural remnants and for emulsions in the presence of apolipoprotein E, the uptakes were 6.7 and 5.8%/min, respectively, close to the physiological rate.

When the perfusion medium contains erythrocytes, the mass of erythrocytes may exceed the liver mass (28,29), and cholesterol present in the erythrocyte membranes may transfer to added emulsions. In our studies, erythrocytes were absent to avoid this complication and, as shown previously, the liver viability was physiological (28–30).

Our findings provide further evidence that apolipoprotein B_{48} is not implicated in the uptake process of chylomicron remnants by the liver. Lipoproteins that contain apolipoprotein B_{48} but not E bind very poorly to receptors (35). Further, the metabolism of chylomicrons and remnants can be reproduced by emulsions entirely lacking in apolipoprotein B_{48} (20,21). Confirming results with very low-density lipoproteins (36), apolipoprotein B is neither necessary nor sufficient for receptor binding, whereas apolipoprotein E is essential. Similarly, the changes in lipid composition of remnants are a necessary but not a sufficient reason to account for remnant-like physiological behavior.

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REFERENCES

1. Redgrave, T.G. (1970) *J. Clin. Invest. 49*, 465–471.
2. Redgrave, T.G., and Martin, G. (1977) *Atherosclerosis 28*, 69–80.
3. Noel, S., Dolphin, P.J., and Rubinstein, D. (1975) *Biochem. Biophys. Res. Commun. 64*, 764–772.
4. Felts, J.M., Itakura, J., and Crane, R.T. (1975) *Biochem. Biophys. Res. Commun. 66*, 1467–1475.
5. Cooper, A.D. (1977) *Biochim. Biophys. Acta 488*, 464–474.
6. Cooper, A.D., and Yu, P.Y.S. (1978) *J. Lipid Res. 19*, 635–643.
7. Sherrill, B.C., and Dietschy, J.M. (1978) *J. Biol. Chem. 253*, 1859–1867.
8. Windler, E., Chao, Y., and Havel, R.J. (1980) *J. Biol. Chem. 255*, 5474–5480.

9. Windler, E.E.T., Kovanen, P.T., Chao, Y.S., Brown, M.S., Havel, R.J., and Goldstein, J.L. (1980) *J. Biol. Chem. 255,* 10464-10471.
10. Sherrill, B.C., Innerarity, T.L., and Mahley, R.W. (1980) *J. Biol. Chem. 255,* 1804-1807.
11. Borensztajn, J., and Kotlar, T.J. (1984) *Proc. Natl. Acad. Sci. USA 81,* 5863-5866.
12. Jones, A.L., Hradek, G.T., Hornick, C., Renaud, G., Windler, E.E.T., and Havel, R.J. (1984) *J. Lipid Res. 25,* 1151-1158.
13. Mahley, R.W., Hui, D.Y., Innerarity, T.L., and Weisgraber, K.H. (1981) *J. Clin. Invest. 68,* 1197-1206.
14. Mjös, O.D., Faergeman, R.L., Hamilton, R.L., and Havel, R.J. (1975) *J. Clin. Invest. 56,* 603-615.
15. Imaizumi, K., Fainaru, M., and Havel, R.J. (1978) *J. Lipid Res. 19,* 712-722.
16. Jeffrey, F., and Redgrave, T.G. (1982) *J. Lipid Res. 223,* 154-160.
17. Windler, E., Chao, Y., and Havel, R.J. (1980) *J. Biol. Chem. 255,* 8303-8307.
18. Shelburne, F.J., Hanks, W.C., Meyers, W.C., and Quarfordt, S.H. (1980) *J. Clin. Invest. 65,* 652-658.
19. Kortz, W.J., Schirmer, B.D., Mansbach, C.M., Shelburne, F., Toglia, M.R., and Quarfordt, S.H. (1984) *J. Lipid Res. 25,* 799-804.
20. Redgrave, T.G., and Maranhao, R.C. (1985) *Biochim. Biophys. Acta 835,* 104-112.
21. Maranhao, R.C., Tercyak, A.M., and Redgrave, T.G. (1986) *Biochim. Biophys. Acta 875,* 247-255.
22. Sripada, P.K. (1986) *J. Lipid Res. 27,* 352-353.
23. Redgrave, T.G., and Carlson, L.A. (1979) *J. Lipid Res. 20,* 217-229.
24. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) *J. Clin. Invest. 34,* 1345-1353.
25. Scanu, A.M., and Edelstein, C. (1971) *Anal. Biochem. 44,* 576-588.
26. Wetterau, J.R., and Jonas, A. (1982) *J. Biol. Chem. 257,* 10961-10966.
27. Shelburne, F.A., and Quarfordt, S.H. (1977) *J. Clin. Invest. 60,* 944-950.
28. Brunengraber, H., Boutry, M., and Lowenstein, J.M. (1973) *J. Biol. Chem. 248,* 2656-2669.
29. Brunengraber, H., Boutry, M., Daikuhara, Y., Kopelovich, L., and Lowenstein, J.M. (1975) *Methods Enzymol. 35,* 597-607.
30. Robins, S.J., and Brunengraber, H. (1982) *J. Lipid Res. 23,* 604-608.
31. Hui, D.Y., Innerarity, T.L., and Mahley, R.W. (1981) *J. Biol. Chem. 256,* 5646-5655.
32. Kita, T., Goldstein, M.S., Brown, M.S., Watanabe, Y., Hornick, C.A., and Havel, R.J. (1982) *Proc. Natl. Acad. Sci. USA 79,* 3623-3627.
33. Fainaru, M., and Deckelbaum, R.J. (1979) *FEBS Lett. 97,* 171-174.
34. Guo, L.L.S., Hamilton, R.L., Goerke, J., Weinstein, J.M., and Havel, R.J. (1980) *J. Lipid Res. 21,* 993-1003.
35. Hui, D.Y., Innerarity, T.L., Milne, R.W., Marcel, Y.L., and Mahley, R.W. (1984) *J. Biol. Chem. 259,* 15060-15068.
36. Bradley, W.A., and Gianturco, S.H. (1986) *J. Lipid Res. 27,* 40-48.

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Effect of Essential Fatty Acid Deficiency on the Size and Distribution of Rat Plasma HDL[1]

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Rat plasma high density lipoproteins (HDL) are comprised of two major particle size subpopulations, HDL_1 (255 Å–140 Å) and HDL_2 (140 Å–84 Å), in which the proportion of arachidonate in fatty acids of cholesteryl esters is greater than 50%. To determine whether decreased availability of arachidonate for cholesterol esterification would alter the distribution and/or amounts of the HDL subpopulations, we compared HDL subpopulations in EFA-deficient and control rats. To separate the effects of EFA deficiency and fat deficiency and to evaluate effects of different saturated fats, we used EFA-deficient diets that were fat-free or that contained 5% saturated fat. The control diets were the EFA-deficient diets plus 1% safflower oil. The saturated fats were hydrogenated coconut oil, hydrogenated cottonseed oil and saturated medium-chain triglycerides. All EFA-deficient diets decreased the proportion of the HDL_1 subpopulation and the peak diameter of the HDL_2 subpopulation. These changes appeared after quite brief EFA depletion in young rats and may be related to the increased liver cholesteryl ester concentrations typical of EFA-deficient rats.
Lipids 23, 106–114 (1988).

The major part of plasma cholesteryl esters (CE) in the rat are carried in high density lipoproteins (HDL), which consist of two major subpopulations, often termed HDL_1 and HDL_2 (1–4). Ultracentrifugal fractionation of the HDL has shown that the HDL_1 has both a lower density and larger particle size (255 Å–140 Å) than the HDL_2 subpopulation (140 Å–84 Å) (5–9). Both subpopulations contain apolipoprotein (apo) A-I, but only the HDL_1 contains apolipoprotein E (1–3,7–9).

In both subpopulations, the proportion of arachidonate in fatty acids of CE is greater than 50% (1), a fact that shows the importance of arachidonate in the transport of cholesterol and conversely the importance of cholesterol in the transport of arachidonate in the rat (10,11). Therefore, one might speculate that a change in the availability of arachidonate for cholesterol esterification would alter the distribution and/or amounts of the HDL subpopulations. Information on such changes is needed to understand the role of essential fatty acids (EFA) in plasma cholesterol metabolism.

Narayan and coworkers obtained evidence to support the idea that lack of EFA changes the distribution of HDL subpopulations. Narayan and McMullen (12) reported that EFA-deficient rats fed a fat-free diet had a lower concentration and proportion of plasma HDL in the fraction corresponding to the HDL_1 subpopulation than did rats fed a diet containing 4% corn oil. However, the level of dietary fat and the type of dietary fat affect the composition and distribution of HDL in rats fed diets adequate in EFA (13). Thus, the comparison of a fat-free diet with a 4% corn oil diet did not resolve whether lack of EFA or lack of fat caused the changes in HDL distribution observed by Narayan and McMullen. Earlier work (14,15) implied that the reduction in the HDL_1 subpopulation was an effect of EFA deficiency because this subpopulation was reduced in rats fed a 10% hydrogenated coconut oil diet (14) in comparison with rats fed a 10% corn oil diet in a previous experiment (15).

To distinguish more definitely the effects of EFA deficiency from those of fat deficiency and to evaluate how saturated fats of different fatty acid composition might influence HDL distribution in both EFA-deficient and control rats, we have investigated the distribution of HDL subpopulations in EFA-deficient rats fed diets that were fat-free or that contained 5% saturated fat, in comparison with rats fed these EFA-deficient diets supplemented with 1% safflower oil (SAFF). The saturated fats used were hydrogenated coconut oil (HCNO), hydrogenated cottonseed oil (HCSO) and saturated medium-chain triglycerides (MCT). Our results show that all of the EFA-deficient diets produced a large decrease in the proportion of the HDL_1 subpopulation.

MATERIALS AND METHODS

Animals and diets. Male weanling Sprague-Dawley rats (Bantin-Kingman, Fremont, CA) weighing 45–50 g at 21 days of age were assigned to diets so that the average initial body weights of each group were equal. Pairs of rats were housed in suspended, wire-bottom cages and given food and tap water ad libitum. The room was kept at 22 C with constant lighting (7 a.m. to 7 p.m.). Body weights and food intakes were recorded three times weekly to monitor growth. Table 1 shows the composition of the purified diets (16,17). EFA-deficient diet groups and respective controls were 0% fat, 1% safflower oil (SAFF); 5% hydrogenated coconut oil (HCNO), 5% HCNO + 1% SAFF; 5% hydrogenated cottonseed oil (HCSO), 5% HCSO + 1% SAFF; 5% medium-chain triglycerides (MCT), 5% MCT + 1% SAFF. A 5% corn oil (CORN) diet was fed to another EFA-adequate group. After 3, 4, 8, 12 or 13 weeks on the diet, rats were fasted for 16 hr (5 p.m.–9 a.m.) before receiving 50 mg sodium pentobarbital/kg body weight intraperitoneally (Nembutal, Abbott Laboratories, North Chicago, IL). Blood was collected by heart puncture.

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Abbreviations: ACAT: acylcoenzyme A:cholesterol acyltransferase; apo: apolipoprotein; CE: cholesteryl esters; EDTA: ethylene diamine tetraacetic acid; EFA: essential fatty acids; HCNO: hydrogenated coconut oil; HCSO: hydrogenated cottonseed oil; HDL: high density lipoproteins; LCAT: lecithin: cholesterol acyltransferase; LDL: low density lipoproteins; MCT: medium-chain triglycerides; PAGGE: polyacrylamide gradient gel electrophoresis; SAFF: safflower oil; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; VLDL: very low density lipoproteins.

TABLE 1

Composition of Purified Diets

Diets[a]	Components, g/100 g of diet: Fat	Sucrose[b]
EFA-deficient		
0% Fat	0	74.5
5% Saturated fat (5% HCNO[c], 5% HCSO[d], or 5% MCT[e])	5	69.5
EFA-adequate		
1% SAFF[f]	1	73.5
5% Saturated fat[c,d,e] + 1% SAFF	6	68.5
5% Corn[g]	5	69.5

[a]All diets included (g/100 g diet): 20.0 vitamin-free casein; 91.0% crude protein (Teklad, Madison, WI); 1.0 B-vitamin mix in sucrose (16) (vitamins from Sigma Chemical Co., St. Louis, MO); 1.0 vitamin A, D, E mix in sucrose (16) (vitamins from Sigma); 3.5 mineral mix UCB-1Rb (17); Na_2SeO_3 and $KCr(SO_4)_2 \cdot 12H_2O$ added separately in sucrose to give 0.1 mg Se and 2 mg Cr/kg diet (Mallinckrodt, Paris, KY); 0.18 choline bitartrate (Sigma).

[b]Powdered sucrose, C & H Sugar Co. (San Francisco, CA).

[c]Hydrogenated coconut oil, California Fats and Oils (Richmond, CA). HCNO fatty acids, % by weight: 8:0, 4%; 10:0, 6%; 12:0, 49%; 14:0, 18%; 16:0, 10%; 18:0, 12%.

[d]Hydrogenated cottonseed oil, Wilsey Foods (San Francisco, CA). HCSO fatty acids, % by weight: 16:0, 25%; 18:0, 71%; 18:1n-9, 2%; 18:2n-6, 0.5%; with 1.5% minor unidentified compounds.

[e]Medium-chain triglyceride oil, Mead Johnson (Evansville, IN). MCT fatty acids, % by weight: 8:0, 67%; 10:0, 23%; with 6% of fatty acids of chain length shorter than C_6 and 4% longer than C_{10}.

[f]Safflower oil, Saffola (Los Angeles, CA). SAFF fatty acids, % by weight: 16:0, 7%; 18:0, 3%; 18:1n-9, 12%; 18:2n-6, 77%; 18:3n-3, 0.5%.

[g]Corn oil, Mazola, Best Foods (Englewood Cliffs, NJ). Corn oil fatty acids, % by weight: 16:0, 13%; 16:1n-7, 0.1%; 18:0, 2.3%; 18:1n-9, 26.1%; 18:2n-6, 58%; 18:3n-3, 1.1%.

Lipoprotein fractionation. Blood containing 1 mg Na_2EDTA/ml was spun at 2500 rpm (1223 × g) for 30 min at 4 C (IEC Centrifuge model PR-2, International Equipment Co., Boston, MA) to obtain plasma. For isolation of lipoproteins of d ⩽ 1.20 g/ml, 2 ml plasma were mixed with 4 ml salt solution (0.194 M NaCl, 4.506 M NaBr, d = 1.310 g/ml) to a final density of 1.217 g/ml. Samples were ultracentrifuged at 40,000 rpm (143,000 × g) for 24 hr at 15 C (Beckman 40.3 rotor, centrifuge model L2-65B, Beckman Instruments, Palo Alto, CA) (18). The top 1 ml fraction of each sample (d ⩽ 1.20 g/ml), which contained the total plasma lipoproteins, was stored at 4 C and electrophoresed within one wk. Fraction density was checked at 26 C with a Bausch and Lomb Abbe-3L Refractometer (Bausch and Lomb, Rochester, NY).

To isolate the HDL within the d 1.04–1.20 g/ml fraction, 4 ml rat plasma (from individual rats or equal volumes of plasma pooled from each of four rats) containing 10 mM phenylmethylsulfonyl fluoride were mixed with 2 ml salt solution (0.194 M NaCl, 0.479 M NaBr, d = 1.043 g/ml) and ultracentrifuged at 40,000 rpm (143,000 × g) for 24 hr at 15 C in a Beckman 40.3 rotor. The top 1 ml (d ⩽ 1.04 g/ml) was removed, and 1.230 g NaBr was added to the subnatant. The tubes were ultracentrifuged again at 40,000 rpm for 24 hr at 15 C. The top 1 ml fractions (d 1.04–1.20 g/ml) were stored at 4 C and electrophoresed within one wk.

Polyacrylamide gradient gel electrophoresis (PAGGE). Lipoproteins of the d ⩽ 1.20 g/ml top 1 ml fraction were electrophoresed through 4–30% or 2–16% polyacrylamide gradient slab gels (PAA 4/30, Pharmacia, Piscataway, NJ) at 125 V, constant voltage, at 10 C for 24 hr (Pharmacia Electrophoresis GE-2/4 Apparatus; LKB 2197 Power Supply, 2209 MultiTemp, LKB, Gaithersburg, MD) according to Nichols et al. (19) except that internal standard was omitted. Proteins were fixed in 10% sulfosalicylic acid for one hr and stained with 0.2% Coomassie blue R-250 in 50% methanol/10% acetic acid for 1.5 hr. Gels were destained in 7.5% acetic acid/5% methanol until background was completely clear.

Densitometry. An automated RFT densitometer (Transidyne General, Ann Arbor, MI) scanned the gels at 530 nm wavelength. A PDP8 computer (Digital Equipment Corp., Maynard, MA) calculated scan data, and a hard copier (Model 4631, Tektronix Inc., Beaverton, OR) recorded data displayed on a terminal (Model 4012, Tektronix). To calibrate particle size, the computer program generated a standard curve of peak R_f values vs logarithm of Stokes' radii of the reference proteins. R_f is the ratio of migration distances of a specific protein peak and of the peak of the furthest migrating standard protein in the same gel. Reference proteins (and radii in Å) were thyroglobulin (85.0), apoferritin (61.0), lactate dehydrogenase (40.8), and bovine serum albumin (35.5). Rat HDL peak R_f values were converted to particle diameters, and areas under the densitometric scans were integrated (19). The R_f ranges of the rat plasma HDL_1 subpopulation (0.16–0.40; corresponding to 255 Å–140 Å) and of the rat plasma HDL_2 (0.40–0.75; corresponding to 140 Å–84 Å) were assigned by inspection of the densitometric tracings.

SDS-PAGE of apolipoproteins. Plasma lipoproteins of the d ⩽ 1.20 g/ml top fraction were dialyzed 18 hr at 4 C against 0.05 M NaCl, 0.001 M Na_2EDTA, pH 6–8. Protein content was assayed by the method of Lowry et al. (20) using bovine serum albumin (Fraction V, Sigma) as standard. Samples of 20–30 μg protein were denatured by boiling for 15 min in 1% sodium dodecyl sulfate (SDS), with or without 5% 2-mercaptoethanol, and electrophoresed through 10% polyacrylamide tube gels (21) at constant current of 10 mA per tube for five hr at 16 C in the Weber and Osborn continuous buffer system (22). SDS-PAGE gels were processed as noted for polyacrylamide gradient gels and scanned densitometrically at 603 nm. The PDP8 computer generated a standard curve of peak R_f vs logarithm of molecular weight of the reference proteins. Standard proteins (MW in kilodaltons) were bovine serum albumin (67.0), human apoE (34.0), bovine apoA-I (28.0) and lysozyme (14.4).

Statistics. The average HDL_2 peak diameters of EFA-deficient rats were compared with those of corresponding control rats by Student's unpaired t-test (23).

RESULTS

Distribution of plasma lipoproteins (d ⩽ 1.20 g/ml) in 4–30% polyacrylamide gradient gels. Figures 1–4 show the PAGGE patterns, stained for protein, for individual

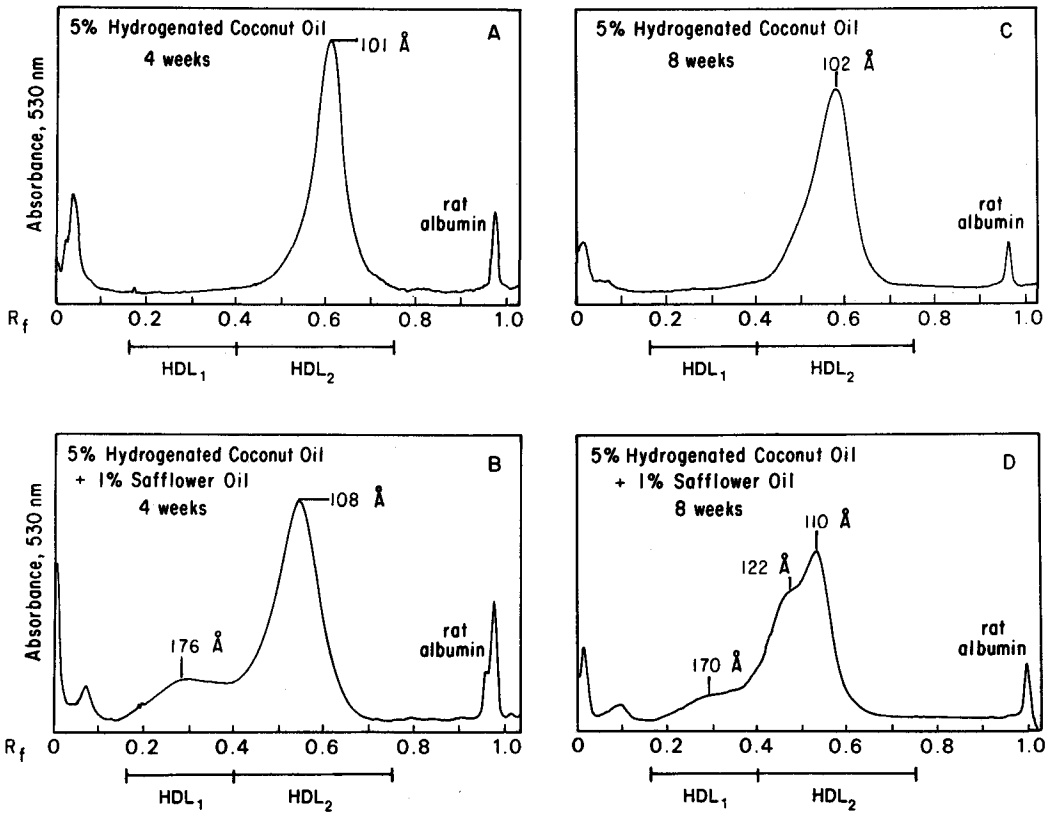

FIG. 1. Representative densitometric scans of 4–30% polyacrylamide gradient electrophoresis gels (PAGGE; Coomassie R-250 protein stain) of plasma HDL (d ≤ 1.20 g/ml fraction) from individual rats on an EFA-deficient 5% hydrogenated coconut oil (HCNO) diet for four or eight wk (A, C) compared with controls (B, D).

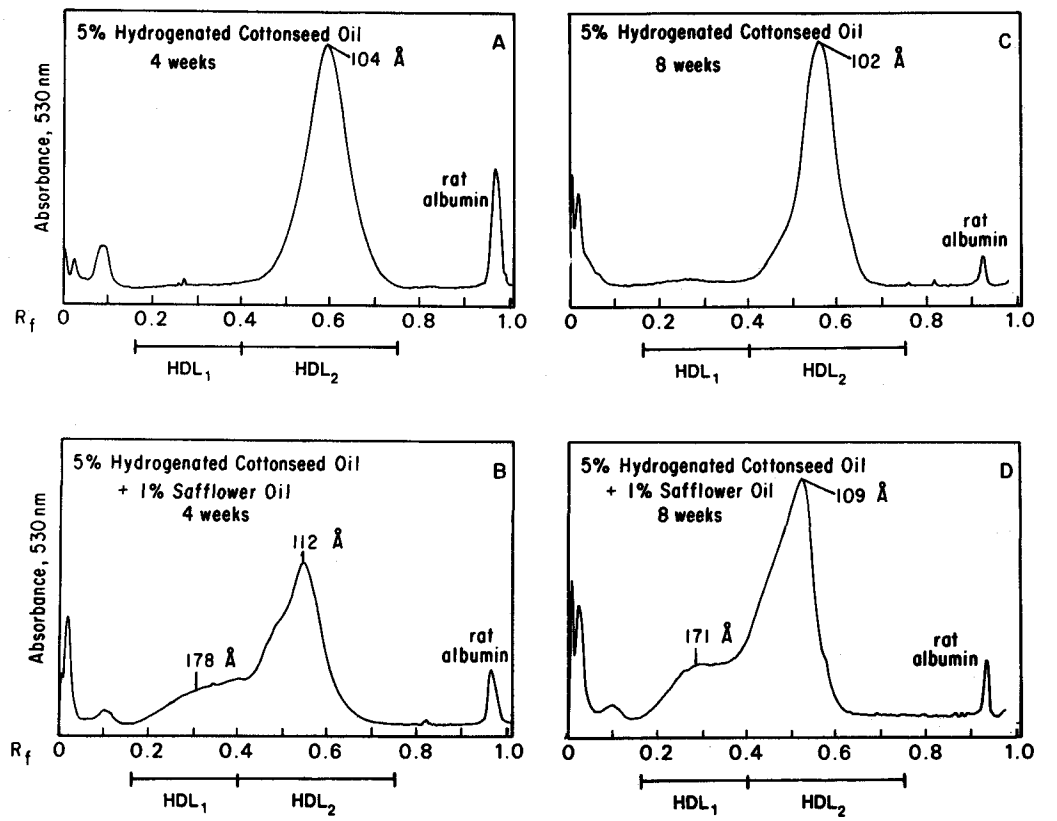

FIG. 2. Representative densitometric scans of 4–30% PAGGE of plasma HDL (d ≤ 1.20 g/ml fraction) from individual rats on an EFA-deficient 5% hydrogenated cottonseed oil (HCSO) diet for four or eight wk (A, C) compared with controls (B, D).

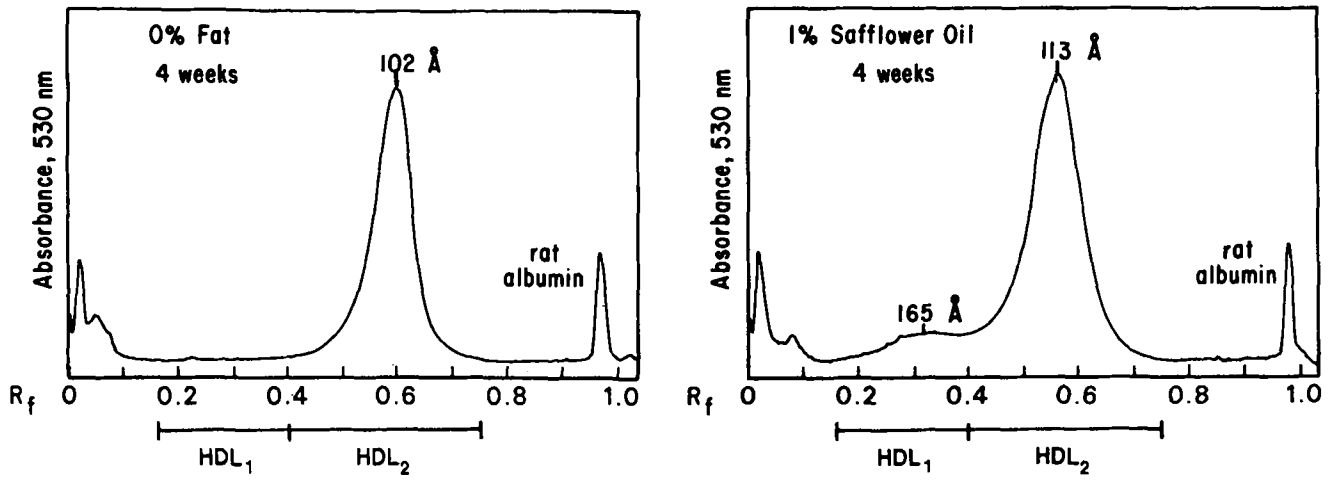

FIG. 3. Representative densitometric scans of 4-30% PAGGE of plasma HDL (d ≤ 1.20 g/ml fraction) from individual rats on an EFA-deficient 0% fat diet for four wk (left) compared with control (right).

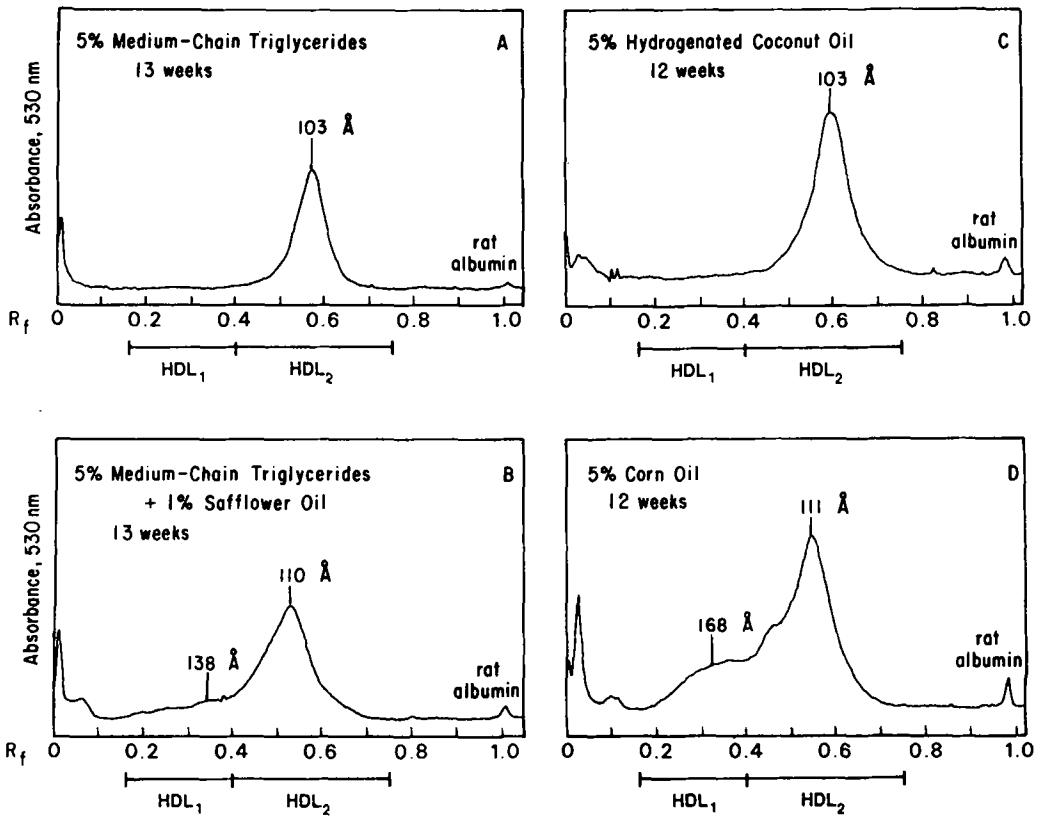

FIG. 4. Representative densitometric scans of 4-30% PAGGE of plasma HDL (d ≤ 1.20 g/ml fraction) from individual rats on a 5% medium-chain triglyceride oil (MCT) diet for 13 wk (A) or 5% HCNO for 12 wk (C) compared with EFA-adequate samples (B, D).

rats fed EFA-deficient and control diets for four wk or more. HDL patterns of rats fed control diets for four wk show significant amounts of material in the HDL_1 as well as in the HDL_2-size intervals. In contrast, the HDL patterns of EFA-deficient rats show a greater predominance of the HDL_2 species and very little material in the HDL_1 interval. The VLDL and LDL patterns of EFA-deficient rats, as evaluated by PAGGE on 2–16% gels (not shown), were very similar to those of EFA-supplemented rats.

A more quantitative evaluation of the 4–30% PAGGE tracings is given in Table 2, which shows the percent contribution of HDL_1 and HDL_2 to the total HDL pattern after three, four or eight weeks of feeding the test diets. In the EFA-deficient groups, the contribution of HDL_1

TABLE 2

Effect of Diet on Percentage of Contribution of HDL Subpopulations to Electrophoretic Profile[a] of Total HDL

Diet group (n)	% of Total HDL area, mean ±SD_{n-1}	
	HDL_1	HDL_2
	Total lipoprotein fraction (d ⩽ 1.20 g/ml)	
HCNO + SAFF 4 wk (11)	20.4 ± 11.1	79.6 ± 11.1
HCNO 4 wk (10)	6.9 ± 3.3§	93.1 ± 3.3*
HCNO + SAFF 8 wk (10)	15.4 ± 3.7	84.6 ± 3.7
HCNO 8 wk (14)	7.2 ± 3.3§	92.8 ± 3.3
HCSO + SAFF 4 wk (6)	21.3 ± 2.7	78.7 ± 2.7
HCSO 4 wk (4)	7.4 ± 1.1§	92.6 ± 1.1§
HCSO + SAFF 8 wk (13)	17.7 ± 5.0	82.3 ± 5.0
HCSO 8 wk (12)	10.1 ± 2.7§	89.9 ± 2.7*
1% SAFF 4 wk (4)	17.1 ± 3.6	82.9 ± 3.6
0% FAT 4 wk (4)	7.0 ± 0.6§	93.0 ± 0.6*
	HDL subfraction (d 1.04–1.20 g/ml)	
HCNO + SAFF 3 wk (4)	16.5 ± 1.4	83.5 ± 1.4
HCNO 3 wk (5)	5.9 ± 1.6§	94.1 ± 1.6*

[a]4–30% PAGGE.

*Significantly different from corresponding control at $p < 0.05$.

§Significantly different from corresponding control at $p < 0.01$.

TABLE 3

Effect of Diet on Diameter* of Rat Plasma HDL_2 Subpopulation

Diet group	n	HDL diameter, Å (X ± SD_{n-1})
Three weeks on diet		
5% HCNO + 1% SAFF[a]	4	108.6 ± 1.0
5% HCNO[a]	5	100.0 ± 0.9§
Four weeks on diet		
5% HCNO + 1% SAFF	14	109.9 ± 2.0
5% HCNO	14	102.2 ± 1.5§
5% HCSO + 1% SAFF	10	114.0 ± 3.7
5% HCSO	9	102.9 ± 3.6§
1% SAFF	6	107.7 ± 1.9
0% FAT	6	100.9 ± 1.1§
Eight weeks or more on diet		
5% HCNO + 1% SAFF[b]	11	109.9 ± 2.2
5% HCNO[b]	13	101.9 ± 2.5§
5% HCSO + 1% SAFF[b]	13	107.8 ± 1.8
5% HCSO[b]	12	102.0 ± 3.4§
5% MCT + 1% SAFF[c]	2	109.8 ± 0.6
5% MCT[c]	2	100.4 ± 3.0
5% CORN[d]	2	112.0

*Determined by 4–30% PAGGE.

§Significantly different from corresponding control at $p < 0.01$.

[a]d 1.04–1.20 g/ml HDL subfraction samples; all others were d ⩽ 1.20 g/ml HDL subfractions.

[b]Eight wk on diet.

[c]Thirteen wk on diet.

[d]Twelve wk on diet.

to the HDL pattern is only half (7–10%) that observed in the corresponding control groups (15–21%). These differences are statistically significant. The proportion of HDL_1 to total HDL in the fat-free diet group (7.0% of HDL pattern area) is similar to the HDL_1 proportions in the EFA-depleted 5% HCNO and 5% HCSO diet groups (6.9% and 7.4% of HDL pattern area, respectively). Thus, this change results from EFA deficiency, not from fat deficiency.

Table 3 shows the mean particle diameter determined by 4–30% PAGGE at peak maximum of the HDL_2 subpopulation from deficient and control rats. In all of the deficient groups, the mean HDL_2 diameter ranges from 100.0 to 102.9 Å. These values are significantly smaller (by 6–11 Å) than the mean diameters of the HDL_2 for the corresponding control groups.

In certain of the dietary studies, HDL were isolated in the density range of d 1.04–1.20 g/ml in order to reduce any contribution of LDL to the large particle end of the HDL pattern. Figure 5 shows the PAGGE distribution of the d 1.04–1.20 g/ml lipoproteins from rats fed the 5% HCNO diet and the 5% HCNO + 1% SAFF diet for three and four weeks. In comparison with controls fed the 5% HCNO + 1% SAFF diet, the changes in HDL subpopulation distribution and particle diameter in this density fraction from EFA-deficient rats are similar to the changes observed with the d ⩽ 1.20 g/ml lipoproteins (Tables 2 and 3). The changes in the d 1.04–1.20 g/ml fraction were evident in rats fed the 5% HCNO diet for only three wk (samples from individual rats) and were more pronounced after four wk (pooled plasma samples).

Plasma apolipoproteins separated by SDS-PAGE. Figures 6 and 7 show SDS-PAGE tube gels and densitometric tracings for the apoproteins of the total plasma

TABLE 4

Percentage of Contribution of Major Plasma Apolipoproteins to SDS-PAGE Profiles*

Diet (4 wk)	apoE	apoA-I	apoA-IV
	as % of scanning units (apoE + apoA-I + apoA-IV)		
1% SAFF	23.5	69.7	6.9
0% FAT	3.8	91.3	4.9
5% HCNO + 1% SAFF	17.2	75.0	7.8
5% HCNO	5.9	85.4	8.7

*Determined by analysis of 10% polyacrylamide-1% SDS tube gels (Fig. 6).

lipoprotein d ⩽ 1.20 g/ml fraction from individual rats fed the fat-free diet, the 1% SAFF diet, the 5% HCNO diet and the 5% HCNO + 1% SAFF diet. The proportion of apoE (expressed as % of the sum of the areas of the apoA-I, apoA-IV and apoE peaks) in the total lipoprotein fraction from EFA-deficient rats is only one-third to one-sixth that of the EFA-supplemented rats (Table 4). This decrease in apoE is consistent with the decreases in both the HDL_1 subpopulation, in which apoE is a major component (1, 7, 8), and the plasma triglyceride concentration in EFA-deficient rats (12,24). Correspondingly, the

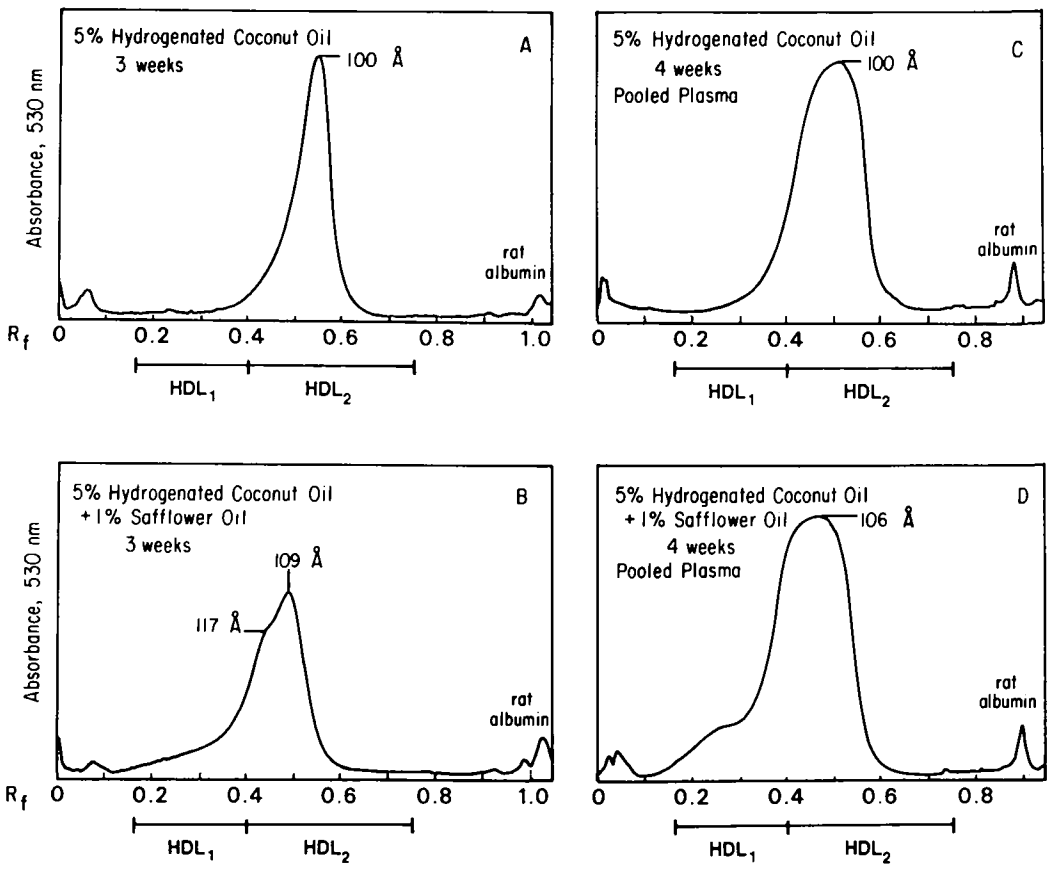

FIG. 5. Representative densitometric scans of 4–30% PAGGE of plasma HDL (d 1.04–1.20 g/ml fraction) from individual rats (A, B) on an EFA-deficient diet (5% HCNO) or control diet after three wk. Scans of HDL from plasma pooled equally from four rats of the same diet groups are shown in C and D.

proportion of the apoA-I peak is greater in samples from EFA-deficient rats than from their corresponding controls. Lastly, the proportion of the apoA-IV band is somewhat greater with the fat-containing diets than with the fat-free diet, an expected result if synthesis of apoA-IV is stimulated by fat feeding (25).

DISCUSSION

Changes in distribution and size of HDL subpopulations. We conclude that the changes in particle size distributions of HDL subpopulations in the EFA-deficient rats are due to a lack of EFA and not to a lack of dietary fat. The changes in particle size distribution also appear independent of the type of saturated fat in the diet, because the effects of EFA-deficiency were similar with diets supplying long chain saturated fatty acids (HCSO) or medium chain fatty acids (HCNO and MCT). All EFA-deficient groups showed a pronounced decrease in the proportion of the HDL_1 subpopulation and a decreased particle diameter of the main peak of the HDL_2 subpopulation in comparison with controls fed the same diet + 1% safflower oil. Furthermore, the distribution and size of the HDL subpopulations were similar in the EFA-deficient groups fed the fat-free diet and in those fed the diets containing 5% saturated fats.

Because the HDL subpopulation distribution and particle size were similar between the fat-free diet group and the groups fed the 5% saturated fat diets, we consider it unlikely that the difference between the control and EFA-deficient groups in HDL subpopulation distribution and particle size resulted from the slightly higher (1%) level of total fat in the control diets, rather than from the presence of EFA. In planning our experiments, we considered it more important to have the same amount of saturated fat in all the deficient and control diets because of evidence that saturated fat increases the need for EFA (26–28). Therefore, in preparing control diets we chose to add 1% safflower oil to the EFA-deficient diets containing 5% saturated fat rather than to maintain a constant total fat level by reducing saturated fat to 4%.

We extended the diet period from four wk to longer periods to test the effects of more pronounced EFA depletion and/or age. We found no additional decreases in the particle size of the HDL_2 subpopulation after eight wk depletion or more (12 and 13 wk, data not shown), despite the somewhat greater tissue EFA depletion (17). EFA depletion for eight wk or even longer generally is used in studies of EFA deficiency in rats to maximize tissue EFA depletion (12,29,30). However, even two weeks of EFA depletion will produce large changes in plasma and liver EFA in young rats (31–33). Thus, if distribution of HDL subpopulations depends on availability of EFA, then it is likely that changes in HDL subpopulation distribution should appear after EFA-depletion periods of two weeks or less. Evidence for such changes has recently been obtained (34).

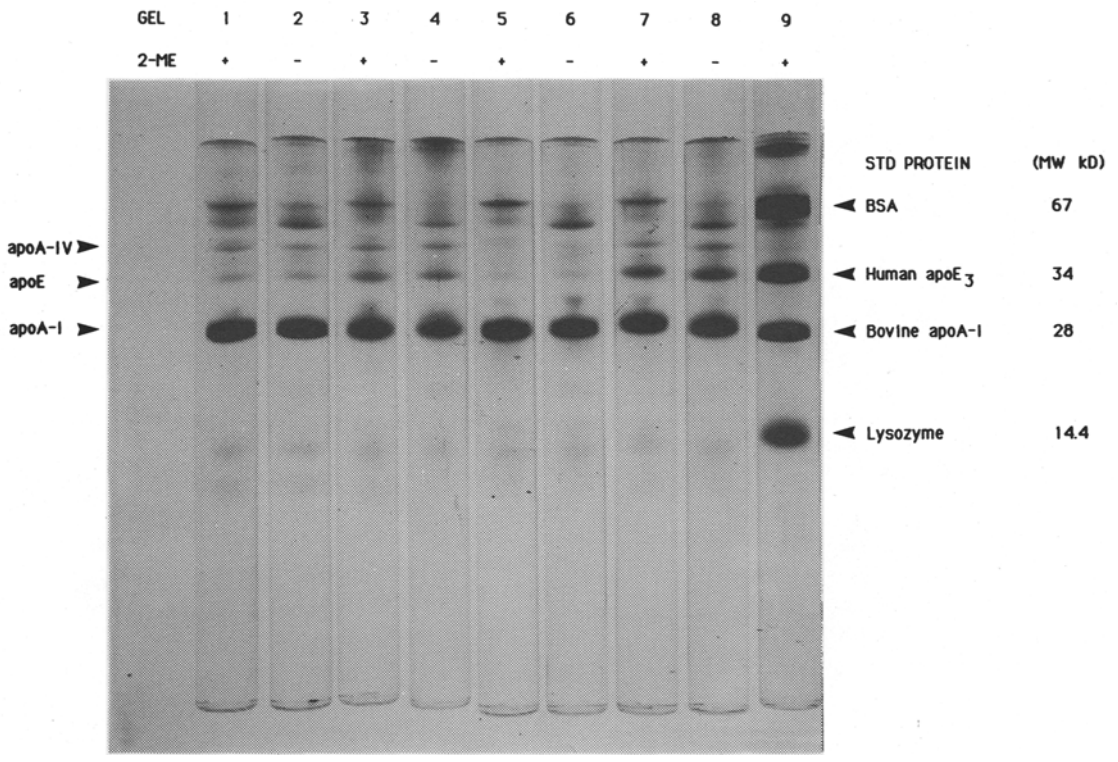

FIG. 6. 10% PAGE-1% SDS tube gels. Rat plasma lipoproteins (d ≤ 1.20 g/ml fraction) were denatured in the presence (+) or absence (−) of 2-mercaptoethanol. Gels 1–8 contain 30 μg protein. Gel 9 contains 55 μg standard protein mixture. 1,2: 5% HCNO diet, four wk. 3,4: (controls) 5% HCNO + 1% SAFF, four wk. 5,6: 0% FAT, four wk. 7,8: (controls) 1% SAFF, four wk.

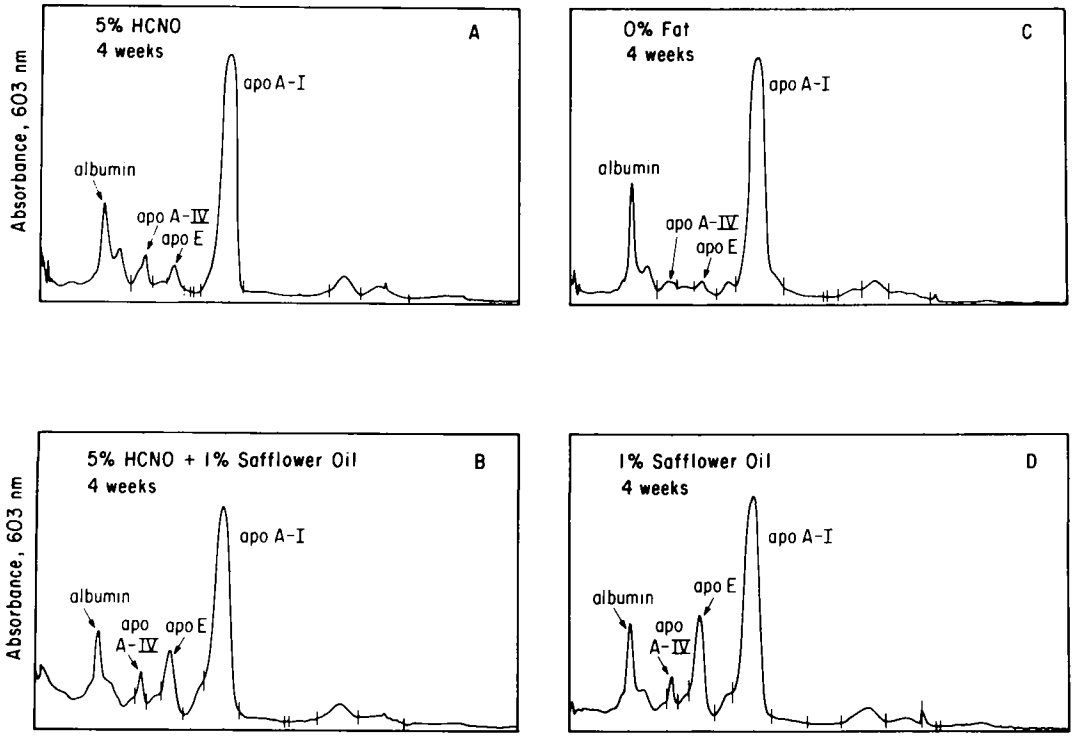

FIG. 7. Densitometric scans of rat plasma apolipoproteins (d ≤ 1.20 g/ml fraction) electrophoresed through 10% PAGE-1% SDS tube gels (see Fig. 6). A (gel 1): 5% HCNO four-week diet. B (gel 3): 5% HCNO + 1% SAFF four-wk control. C (gel 5): 0% FAT four-wk diet. D (gel 7): 1% SAFF four-wk control.

The mean diameter of particles in the HDL_2 subpopulation did not change between four and eight weeks in rats fed the control diets, except for the 5% HCSO + 1% SAFF group. However, with the latter group there was a considerable decrease between four and eight weeks in the mean HDL_2 diameter. This change cannot be attributed to lack of dietary EFA because a level of 1% safflower oil provides linoleate as ca. 1.5% of the food energy, a level that is 50% greater than the minimum EFA requirement. The adequacy of the linoleate intake of the 5% HCSO + 1% SAFF group also is supported by the fact that the levels of linoleate and arachidonate in liver phospholipids of this group are as high as in rats fed the 5% HCNO + 1% SAFF diet or the 5% corn oil diet (35). The size ranges of both the HDL_1 and the HDL_2 subpopulations in our control groups are very similar to values reported by Ha and coworkers, who used PAGGE to measure HDL particle size of rats fed a nonpurified diet (9).

Possible causes of the decrease in the HDL_1 subpopulation. The relative decrease in the HDL_1 subpopulation in the EFA-deficient groups could be caused by its reduced formation and/or increased removal. Reduced formation could occur because of decreased supply of potential precursor materials for HDL_1 production, e.g. VLDL surface components or the HDL_2 subpopulation (3). However, available evidence is not consistent with the possibility of reduced formation. In EFA-deficient rats, VLDL secretion and catabolism are increased, as indicated by increased plasma triglyceride secretion and catabolism (24), and plasma LCAT activity is also increased (29,36, 37). The amount of the HDL_2 subpopulation in deficient rats was similar to that in controls, as indicated by the PAGGE densitometric tracings of equivalent plasma volumes, although we have not made detailed analyses of the HDL subpopulations to determine the proportion and concentration of the lipid and protein components.

There is some evidence to support the possibility of more rapid removal of the HDL_1 subpopulation. This evidence includes increased hepatic uptake of radiolabeled plasma cholesterol (37) and increased concentrations of liver CE in EFA-deficient rats (24,35,38,39), despite decreased liver cholesterol synthesis in deficient rats (38,39). Increased liver CE, of course, could arise from other mechanisms, including increased hepatic ACAT activity (37), decreased biliary secretion of cholesterol and bile acids (40,41) or increased hepatic lipase activity (42,43). Hepatic ACAT activity is increased in EFA-deficient rats (37) but bile secretion and composition in EFA-deficient rats have not been tested critically (40). We have found no reports on hepatic triglyceride lipase activity in EFA-deficient rats. Yet, if hepatic lipase activity were increased in EFA deficiency, such an increase could result in greater transfer of cholesterol from HDL_1 to the liver, where HDL_1 cholesterol may be a preferred substrate for bile acid synthesis (44,45). The decreased hepatic cholesterol synthesis in EFA-deficient rats (38,39) also might lead to a compensatory increase in extrahepatic cholesterol synthesis (40) and reverse cholesterol transport (46). Future experiments should measure reverse cholesterol transport, hepatic triglyceride lipase activity, and biliary bile acid and cholesterol excretion as EFA deficiency develops. These measurements would show whether changes in these processes precede and lead to the increase in liver CE and the decrease in the HDL_1 subpopulation in EFA-deficient rats.

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REFERENCES

1. Oschry, Y., and Eisenberg, S. (1982) *J. Lipid Res. 23,* 1099-1106.
2. Jansen, H., Schoonderwoerd, G.C., and Dallinga-Thie, G.M. (1983) *Biochim. Biophys. Acta 754,* 271-278.
3. Eisenberg, S. (1984) *J. Lipid Res. 25,* 1017-1058.
4. Dudacek, W.E., and Narayan, K.A. (1966) *Biochim. Biophys. Acta 125,* 604-607.
5. Faloona, G.R., Stewart, B.N., and Fried, M. (1969) *Arch. Biochem. Biophys. 130,* 680-683.
6. Pasquali-Ronchetti, I., Calandra, S., Baccarani-Contri, M., and Montaguti, M. (1975) *J. Ultrastructure Res. 53,* 180-192.
7. Weisgraber, K.H., Mahley, R.W., and Assmann, G. (1977) *Atherosclerosis 28,* 121-140.
8. Lusk, L.T., Walker, L.F., DuBien, L.H., and Getz, G.S. (1979) *Biochem. J. 183,* 83-90.
9. Ha, Y.C., Chang, L.B.F., and Barter, P.J. (1985) *Biochim. Biophys. Acta 833,* 203-210.
10. Chapman, M.J. (1980) *J. Lipid Res. 21,* 789-853.
11. Tinoco, J., Shannon, A., and Lyman, R.L. (1964) *J. Lipid Res. 5,* 57-62.
12. Narayan, K.A., and McMullen, J.J. (1979) *J. Nutr. 109,* 1836-1845.
13. Hostmark, A.T., Spydevold, O., and Lystad, E. (1982) *Lipids 17,* 489-499.
14. Narayan, K.A. (1970) *Cancer Res. 30,* 1185-1191.
15. Narayan, K.A. (1967) *Biochem. J. 103,* 672-676.
16. Williams, M.A., Chu, L.-C., McIntosh, D.J., and Hincenbergs, I. (1968) *J. Nutr. 94,* 377-382.
17. Williams, M.A., Tamai, K.T., Hincenbergs, I., and McIntosh, D.J. (1972) *J. Nutr. 102,* 847-856.
18. Lindgren, F.T. (1975) in *Analysis of Lipids and Lipoproteins* (Perkins, E.G., ed.), pp. 204-224, American Oil Chemists' Society, Champaign, IL.
19. Nichols, A.V., Blanche, P.J., Gong, E.L., and Forte, T.M. (1983) in *CRC Handbook of Electrophoresis* (Lewis, L.A., and Opplt, J.J., eds.), Vol. 3, pp. 29-47, CRC Press, Boca Raton, FL.
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
21. Shore, V.G., Garcia, R.E., Penn, A.L., and Shore, B. (1980) in *CRC Handbook of Electrophoresis* (Lewis, L.A., and Opplt, J.J., eds.), Vol. I, pp. 103-125, CRC Press, Boca Raton, FL.
22. Weber, K., and Osborn, M. (1969) *J. Biol. Chem. 244,* 4406-4412.
23. Snedecor, G.W., and Cochran, W.G. (1967) *Statistical Methods,* Sixth edition, Iowa State University Press, Ames, IA.
24. Bird, M.I., and Williams, M.A. (1982) *J. Nutr. 112,* 2267-2278.
25. DeLamatre, J.G., and Roheim, P.S. (1983) *Biochim. Biophys. Acta 751,* 210-217.
26. Holman, R.T. (1968) *Progr. Chem. Fats Other Lipids 9,* 275-348.
27. Alfin-Slater, R.B., and Aftergood, L. (1968) *Physiol. Rev. 48,* 758-784.
28. Peifer, J.J., and Holman, R.T. (1959) *J. Nutr. 68,* 155-168.

29. Takatori, T., Phillips, F.C., Shimasaki, H., and Privett, O.S. (1976) *Lipids 11,* 272–280.
30. Sano, M., and Privett, O.S. (1980) *Lipids 15,* 337–344.
31. Ostwald, R., Bouchard, P., Miljanich, P., and Lyman, R.L. (1965) *Biochem. J. 97,* 485–499.
32. Lyman, R.L., Ostwald, R., Bouchard, P., and Shannon, A. (1966) *Biochem. J. 98,* 438–450.
33. Stancliff, R.C., Williams, M.A., Utsumi, K., and Packer, L. (1969) *Arch. Biochem. Biophys. 131,* 629–642.
34. Ney, D.M., Ziboh, V.A., and Schneeman, B.O. (1987) *J. Nutr. 117,* 2016–2020.
35. Williams, M.A., Tinoco, J., Hincenbergs, I., and Thomas, B. (1984) *J. Am. Oil Chem. Soc. 61,* 664–665.
36. Privett, O.S., Phillips, F., Shimasaki, H., Nozawa, T., and Nickell, E.C. (1977) *Am. J. Clin. Nutr. 30,* 1009–1017.
37. Sugano, M., and Portman, O.W. (1965) *Arch. Biochem. Biophys. 109,* 312–315.
38. Mukherjee, S., and Alfin-Slater, R.B. (1958) *Arch. Biochem. Biophys. 73,* 359–365.
39. Alfin-Slater, R.B., and Bernick, S. (1958) *Am. J. Clin. Nutr. 6,* 613–624.
40. Turley, S.D., Spady, D.K., and Dietschy, J.M. (1983) *Gastroenterology 84,* 253–264.
41. Davis, R.A., Elliott, T.S., and Lattier, G.R. (1986) *Biochemistry 25,* 1632–1636.
42. Jansen, H., and Hulsmann, W.C. (1985) *Biochem. Soc. Trans. 13,* 24–26.
43. Jansen, H. (1985) *Biochem. Biophys. Res. Commun. 131,* 574–578.
44. Robins, S.J., Fasulo, J.M., Collins, M.A., and Patton, G.M. (1985) *J. Biol. Chem. 260,* 6511–6513.
45. Mackinnon, A.M., Schexnayder, J.A., Sand, T.M., and Davis, R.A. (1985) *Arteriosclerosis 5,* 539a–540a.
46. Davis, R.A., Helgerud, P., Dueland, S., and Drevon, C.A. (1982) *Biochim. Biophys. Acta 689,* 410–414.

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Perfluoro-n-Decanoic Acid: Induction of Peroxisomal β-Oxidation by a Fatty Acid with Dioxin-like Toxicity

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Perfluoro-n-decanoic acid (PFDA) produces toxic effects in rodents similar to those caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin. A single, intraperitoneal dose (50 mg/kg) of PFDA to Sprague-Dawley rats caused disruption of the endoplasmic reticulum, mitochondrial swelling and increases in intracellular lipid droplets in hepatocytes similar to effects reported previously in dioxin toxicity. PFDA treatment led to large decreases in the activity of plasma membrane alkaline phosphodiesterase and mitochondrial cytochrome c oxidase without affecting lysosomal N-acetyl-β-glucoaminidase, endoplasmic reticulum NADPH-cytochrome c reductase or peroxisomal catalase activities. PFDA treatment led to moderate peroxisome proliferation and to very large (20–40-fold) increases in the activity of fatty acyl-CoA oxidase, the rate-limiting enzyme in the peroxisomal system of fatty acid β-oxidation.
Lipids 23, 115–119 (1988).

Perfluorocarboxylic acids and their derivatives are used commercially in applications that take advantage of their surfactant properties, chemical and thermal stabilities and antiwetting actions (1,2). In general, perfluorocarbons are considered to be metabolized very poorly and relatively nontoxic. Perfluoro-n-octanoic acid is moderately toxic with an oral LD50 in Sprague-Dawley rats of about 500 mg/kg (3). Perfluoro-n-decanoic acid (PFDA) is significantly more toxic than the octanoic derivative. The intraperitoneal LD50 in Fisher-344 rats for perfluoro-n-octanoic acid was 189 mg/kg, while that for PFDA was only 41 mg/kg (4,5). Rats dosed with perfluoro-n-octanoic acid died within five days. In contrast, PFDA produced toxic effects very similar to those caused by dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin). Similarities include delayed lethality, decreased food intake, thymic atrophy, bone marrow depression, hepatomegaly and disruption of hepatic architecture (4,5). PFDA differs from dioxin in that it causes severe testicular atrophy and necrosis, and it lacks the extreme species variability in toxic potency characteristic of dioxin. However, there are minor differences in PFDA toxicity between species and strains. For example, the intraperitoneal LD50 of PFDA in Fisher-344 rats is 41 mg/kg and in Sprague-Dawley rats is about 75 mg/kg.

Ultrastructural observations on liver of rats and mice treated with a single dose of PFDA near the LD50 demonstrated peroxisomal proliferation (7). Other agents that induce peroxisomes include the hypolipidemic drug clofibrate, its structural analogs, other hypolipidemic agents structurally unrelated to clofibrate, phthalate esters and acetylsalicylic acid (8). The effects of these compounds on peroxisome proliferation have been assessed by morphological analysis (increase in the number and volume of peroxisomes) or by biochemical analysis (increase in the activity of one or more peroxisomal enzymes). Quantitatively, the two measures of response are not well-correlated. Increases in catalase, the major peroxisomal enzyme, disproportionately may be smaller than the increase in peroxisomal volume. On the other hand, certain peroxisomal enzymes are induced to a much greater extent than catalase. The enzymes of the peroxisomal β-oxidation system are induced readily (8). For example, clofibrate treatment of female rats or bezafibrate treatment of male rats led to large elevations of the peroxisomal fatty acid oxidizing system with no increase in catalase activity and only a slight increase in peroxisomal volume (9).

To examine the hepatic responses to PFDA more completely, we have studied its effects on the activities of a number of liver enzymes and on ultrastructure in male Sprague-Dawley rats. We show here that a single dose of PFDA leads to moderate peroxisome proliferation and causes other morphological effects similar to some of those seen in dioxin toxicity. Biochemically, the most striking effect of PFDA was a very large (20- to 40-fold) increase in peroxisomal fatty acyl-CoA oxidase activity. Thus, perfluoro-n-decanoic acid appears to be the most potent inducer of peroxisomal β-oxidation yet described.

EXPERIMENTAL

Animals and materials. Male, weanling Sprague-Dawley rats were obtained from Harlan Industries (Indianapolis, IN). Rats were housed in individual plastic cages and had free access to water and a commercial, pelleted rat diet. PFDA (nonadecafluorodecanoic acid) was from Aldrich Chemical Co. (Milwaukee, WI). Fatty acyl-CoAs were obtained from P-L Biochemicals (Milwaukee, WI). Other enzyme substrates were from Sigma Chemical Co. (St. Louis, MO).

Biochemical methods. Peroxisomal fatty acyl-coenzyme A oxidase activity was assayed as described (10) using 35 μM palmitoyl-CoA as a substrate. The methods used in the assay of the other enzymes are given in previous publications (10,11). All enzyme assays were conducted on freshly prepared homogenates and were carried out under conditions such that product formation was proportional to the amount of enzyme-containing sample.

Protein was assayed by the method of Lowry et al. (12), and DNA was assayed by the fluorescent dye-binding method of LaBarca and Paigen (13).

Electron microscopy. Sections were dissected from the dorsal caudate lobe of the liver and immediately transferred into a petri dish containing 2.5% glutaraldehyde and 2% formaldehyde fixitive in 0.1 M cacodylate buffer (pH 7.4) in which they were sectioned into small blocks ca. 1 mm cubes. Approximately 20 blocks/animal were placed in small vials containing fixitive. After fixation, they were

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Abbreviation: PFDA, perfluoro-n-decanoic.

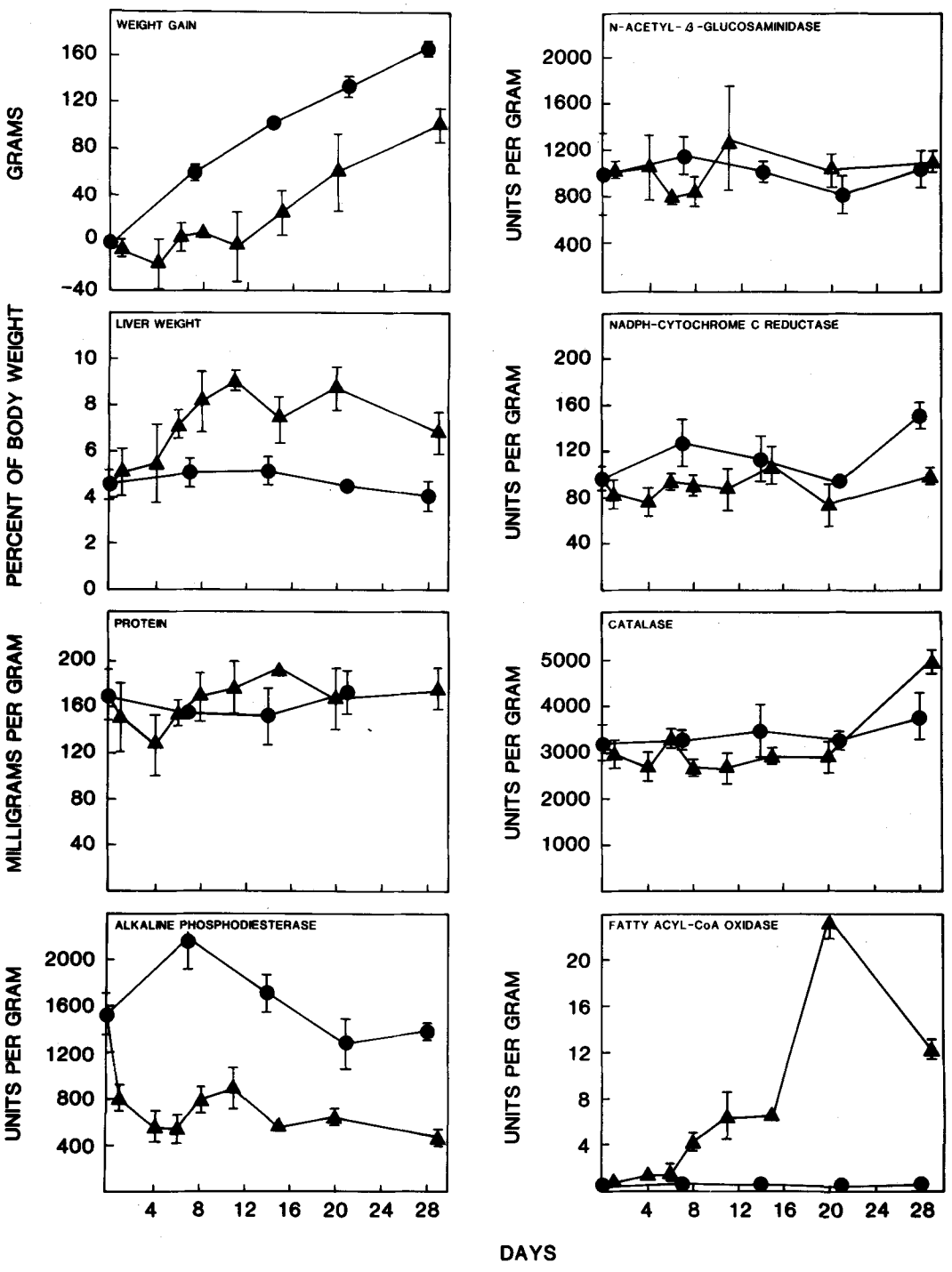

FIG. 1. Effects of a single dose of perfluoro-n-decanoic acid (PFDA) (50 mg/kg) on weight gain, liver weight, and hepatic protein and enzyme contents of male Sprague-Dawley rats. Rats were given a single intraperitoneal dose of PFDA dissolved in propylene glycol (triangles) or the vehicle alone (circles). At each indicated point, three animals were studied. Data are presented as means ± standard deviation for each parameter. Activity of fatty acyl-CoA oxidase is given in units of μmol/min and the activities of other enzymes in arbitrary units of absorbance or change in absorbance under the assay conditions employed.

post-fixed in 2% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Representative thick sections (1 μm) from at least three tissue blocks per animal were mounted on glass slides, stained with toluidine blue and screened by light microscopy to determine areas for thin sectioning. Thin sections (80 nm) were prepared, stained with lead citrate and uranyl acetate, and photographed on a JEOL 100B electron microscope at three different magnifications.

Animal experiments. In experiment 1, rats weighing 150–200 g were given a single intraperitoneal injection of PFDA (50 mg/kg) dissolved in propylene glycol

(approx. 0.4 ml). Note that this dose of PFDA is approximately two-thirds of the LD50 for the Sprague-Dawley rat. Groups of three rats were killed by ether overdose on day 1, 4, 6, 8, 11, 15, 20 or 29. Control rats given the vehicle alone were killed in groups of three on day 0, 7, 12, 21 or 28. One of the 24 PFDA-treated rats died before the scheduled time of death. Livers were removed, weighed and homogenized in four volumes of 0.25 M sucrose containing 1 mM EDTA and 0.1% ethanol using a Polytron homogenizer. Homogenates then were assayed for total protein and for the following enzymes localized in the indicated subcellular organelles (14–16): catalase and fatty acyl-CoA oxidase (peroxisomes), alkaline phosphodiesterase (plasma membrane), NADPH-cytochrome c reductase (endoplasmic reticulum), and N-acetyl-β-glucosaminidase (lysosomes).

In experiment 2, 36 rats weighing 180–215 g were injected with PFDA as described above and studied on the days indicated in Figure 2. Nine control rats received the vehicle alone. No rats died before the scheduled time of death. At each time point, the rats were weighed, and their livers were removed and weighed. After dissection of small samples for morphological analysis, the remaining liver was homogenized as described above. The homogenates were then assayed for protein, DNA, cytochrome oxidase (mitochondria) and fatty acyl-CoA oxidase.

RESULTS AND DISCUSSION

In experiment 1 (Fig. 1), PFDA-treated rats showed an immediate cessation of growth persisting for about two weeks, after which body weight increased in parallel with the controls. Liver to body weight ratio of the treated rats increased to twice that of controls and remained elevated throughout the four-week experiment. Neither liver protein nor the activities of NADPH-cytochrome c reductase, catalase or N-acetyl-β-glucosaminidase were affected substantially by PFDA treatment. However, fatty acyl-CoA oxidase activity increased 20- to 40-fold over the controls and was maximal at three to four weeks after dosing. Alkaline phosphodiesterase activity rapidly fell to about 50% of control values and remained depressed thereafter. In experiment 2 (Fig. 2), PFDA treatment again caused cessation of growth and hepatomegaly. Concomitant with the increase in relative liver size was an increase in total hepatic DNA. Hypolipidemic agents (such as nafenopin, tibric acid, WY-14,643, and BR-931) cause an increase in total DNA as part of a primary mitogenic response that in part accounts for the liver growth (17–19). With PFDA, the etiology of the observed increase in DNA is unknown. PFDA treatment also markedly reduced mitochondrial cytochrome oxidase activity. However, as in experiment 1 the most dramatic effect of PFDA was a large increase in the activity of peroxisomal fatty acyl-CoA oxidase. The changes in hepatic enzymes caused by PFDA treatment persisted for as long as 50 days after a single dose (Fig. 2).

The morphological results obtained in experiment 2 are shown in Figs. 3–6. Hepatocytes of PFDA-treated rats at four days after dosing showed severe disruption of the endoplasmic reticulum and dilution of the cytoplasmic matrix (Fig. 4). Mitochondria were essentially normal at

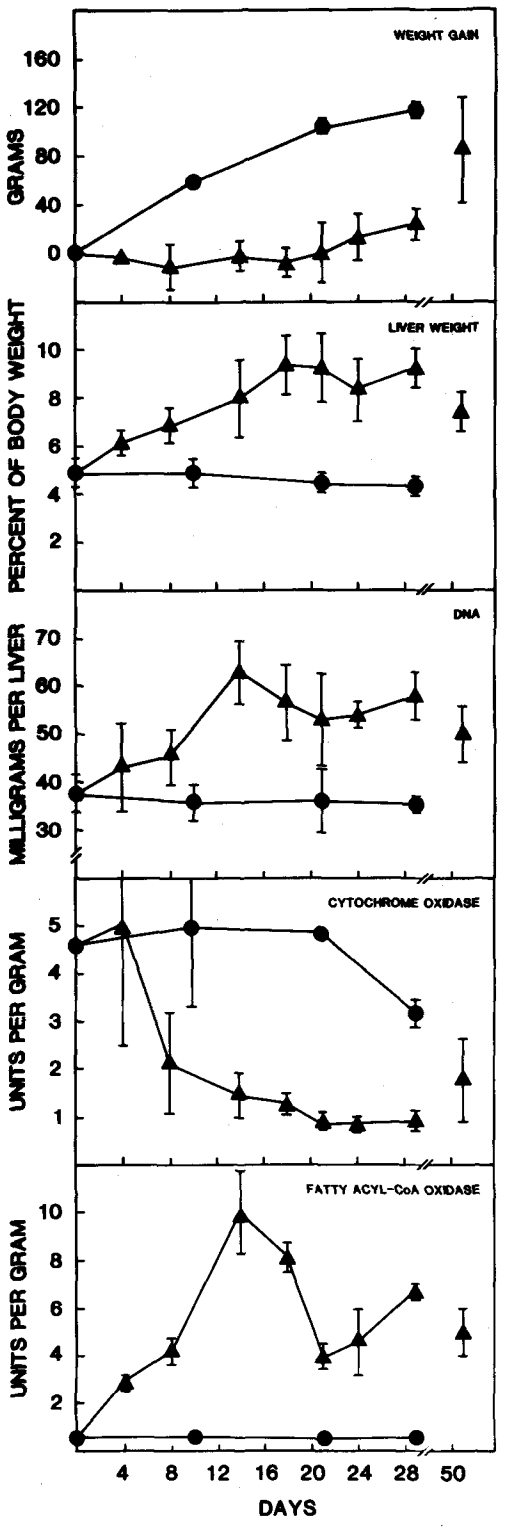

FIG. 2. Effects of a single dose of perfluoro-n-decanoic acid (PFDA) (50 mg/kg) on weight gain, liver weight, and hepatic content of DNA, fatty acyl-CoA oxidase and cytochrome oxidase in male Sprague-Dawley rats. Rats were given a single, intraperitoneal dose of PFDA dissolved in propylene glycol (triangles) or the vehicle alone (circles). At the days indicated, three (days 29 and 51) or five (days 4, 8, 14, 17, 21 and 24) dosed rats and two (days 10, 21 and 29) or three (day 0) control rats were studied. Data are presented as the mean ± standard deviation for each parameter. Activity of fatty acyl-CoA oxidase is given in units of μmol/min and that of cytochrome oxidase in the units previously described (10,11).

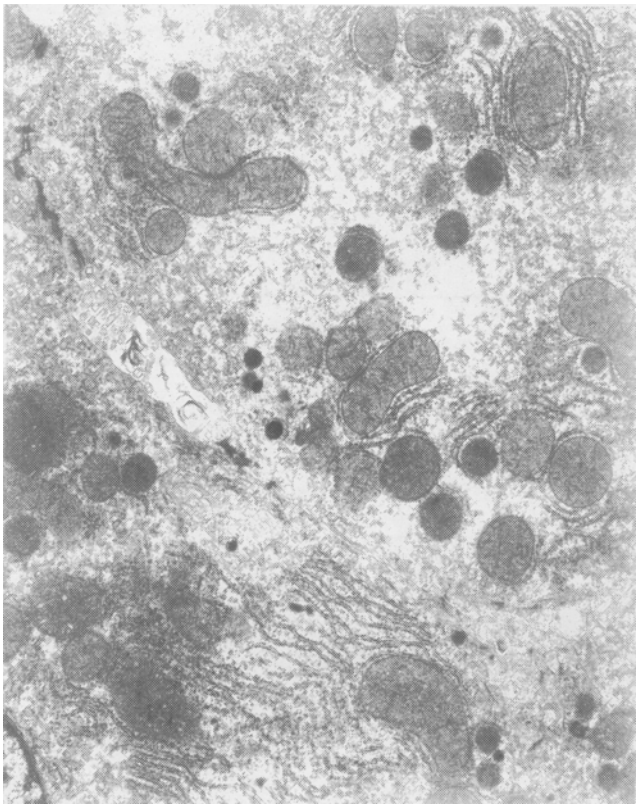

FIG. 3. Ultrastructure of the liver of a control rat (day 0). Note the organization of the cytoplasm and the normal appearance of the mitochondria. A well-developed endoplasmic reticulum particularly is evident at the bottom and upper-right of the field shown. More darkly staining peroxisomes with a dense core are also seen (as, for example, the four dark bodies in the upper-right of the field). Magnification is 8600×.

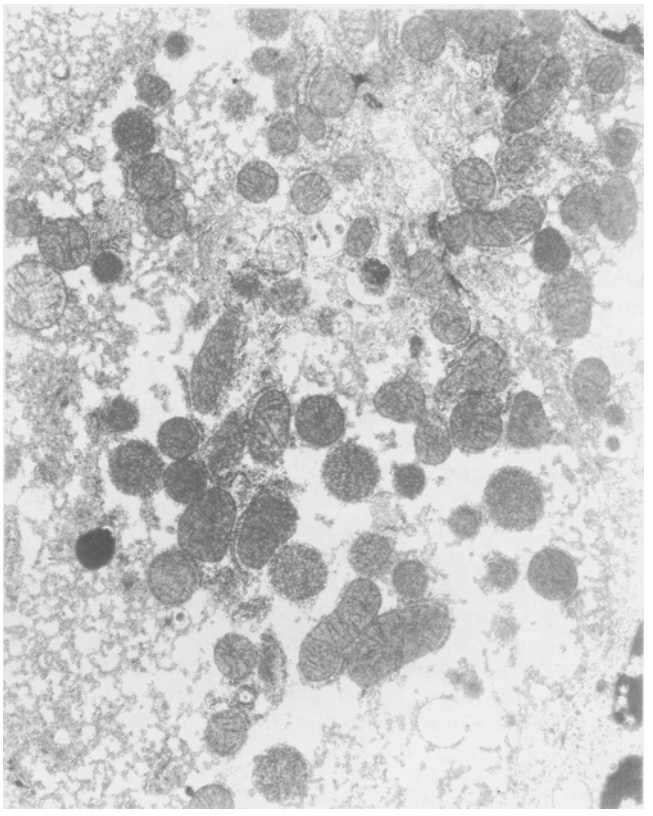

FIG. 4. Ultrastructure of the liver of a PFDA-treated rat (day 4). Note the dilution of the cytoplasmic matrix and the loss on a well-defined endoplasmic reticulum. Mitochondria appear normal. Magnification is 8600×.

this time. At days 14 (Fig. 5), 21 (Fig. 6) and 29 after dosing, the cytoplasmic alterations observed at day 4 remained unresolved. Mitochondria now were swollen, rounded and had lost their cristae. Marked accumulation of cytoplasmic lipid droplets also was observed. Peroxisome proliferation was moderate. In addition, larger, multiple nucleoli were observed in many of the hepatocyte nuclei of PFDA-treated rats. Although the hepatocytes of PFDA-treated rats at days 14 and 21 appear almost necrotic, these animals were not moribund, and most of the treated rats recovered from this dose of PFDA.

With clofibrate, doses from 200–500 mg/kg/day are administered daily for one to three weeks to achieve about a 10-fold induction of fatty acyl-CoA oxidase (8,9). Even for the more potent hypolipidemic drugs, such as bezafibrate, seven daily doses from 25 to 75 mg/kg led to only three- to four-fold increases in hepatic fatty acyl-CoA oxidase activity. With PFDA, a single dose of only 50 mg/kg leads to very large (20- to 40-fold) increases in activity that persist for as long as 50 days after dosing. To our knowledge, PFDA is the most potent inducer of peroxisomal β-oxidation yet described.

The mechanism of fatty acyl-CoA oxidase induction by PFDA is not known. We do know that the compound accumulates in liver (20) but a more complete description of its tissue distribution and pharmacokinetics awaits the completion of studies currently in progress. PFDA is metabolized to constituents more polar than the parent compound (20), and hence it may affect cellular membranes. We also have found that PFDA is an inhibitor of fatty acyl-CoA oxidase activity when added to the in vitro reaction mixture, and it is more potent in this regard than n-decanoic acid is.

While the toxic effects of PFDA resemble those of dioxin, to our knowledge dioxin does not cause peroxisome proliferation or induction of peroxisomal enzymes. However, dioxin does cause other profound changes in hepatic ultrastructure and liver enzymes when administered to rats and mice (21–23). Major ultrastructural changes include proliferation of the endoplasmic reticulum, swelling of mitochondria, increases in the number of autophagic vacuoles and lipid droplets, and loss of continuity of the plasma membrane. Biochemical correlates of these changes include induction of aryl hydrocarbon hydroxylase and a variety of cytochrome P-450 isozymes, increased microsomal lipid peroxidation and reduction in the activity of several plasma membrane enzymes. Thus, some of the effects of dioxin on the liver resemble those that we report here for PFDA, and others do not. Further comparative study of the effects of these two compounds at the cellular and biochemical level may shed light on their respective mechanisms of liver toxicity.

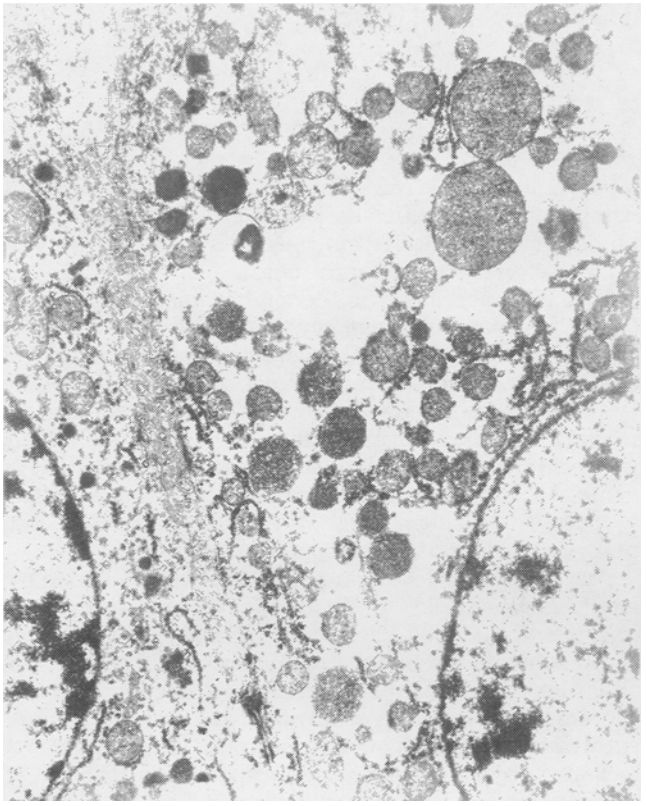

FIG. 5. Ultrastructure of the liver of a PFDA-treated rat (day 14). Cytoplasmic alterations seen on day 4 remained unchanged. In addition, the mitochondria now are swollen and rounded and have lost their cristae (see, for example, the two round, granular bodies in the upper right of the field). Smaller, more darkly staining peroxisomes also are observed. Note also parts of two clear areas in the extreme upper right that represent cytoplasmic lipid droplets. Magnification is 8600×.

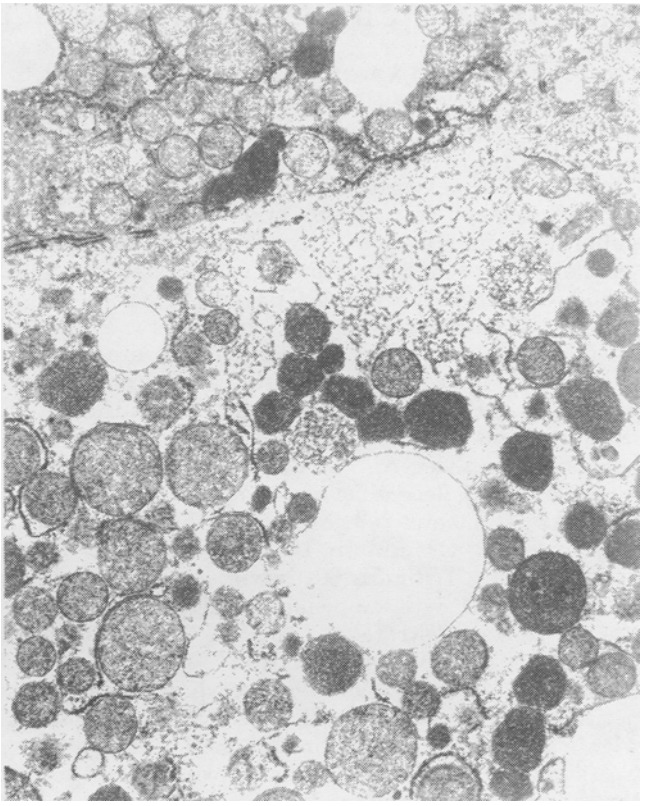

FIG. 6. Ultrastructure of the liver of a PFDA-treated rat (day 21). Mitochondrial and cytoplasmic alterations seen earlier remain unchanged. Note the presence of large lipid droplets (clear, round areas in cytoplasm) and numbers of darkly staining peroxisomes, some of which appear in clusters (center and lower right of the field). Magnification is 8600×.

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REFERENCES

1. Guenthner, R.A., and Vietor, M.L. (1962) *Industrial and Engineering Chemistry Product Research and Development 1,* 165-169.
2. Shinoda, K., and Nomura, T. (1980) *J. Phys. Chem. 84,* 365-369.
3. Griffith, F.D., and Long, J.E. (1980) *Am. Ind. Hyg. Assoc. J. 41,* 576-583.
4. Olson, C.T., and Andersen, M.E. (1983) *Toxicol. Appl. Pharmacol. 70,* 362-372.
5. Olson, C.T., Andersen, M.E., George, M.E., Van Rafelghem, M.J., and Back, A.M. (1982) *Proc. 13th Ann. Conf. on Environ. Toxicol.,* Air Force Aerospace Medical Research Laboratory Technical Report, AFAMRL-TR-82-101, 287-303.
6. Van Rafelghem, M.J., Andersen, M.E., Bruner, R., and Mattie, D. (1982) *The Toxicologist 2,* 148.
7. Van Rafelghem, M.J., Mattie, D.R., Bruner, R.H. and Andersen, M.E. (1987) *Fundam. Appl. Toxicol. 9,* 522-540.
8. Kindl, H., and Lazarow, P.B. (eds.) (1982) *Peroxisomes and Glyoxysomes,* New York Academy of Sciences, New York, NY.
9. Lazarow, P.B., Shio, H., and Leroy-Houyet, M.A. (1982) *J. Lipid Res. 23,* 317-326.
10. Walusimbi-Kisitu, M., and Harrison, E.H. (1983) *J. Lipid Res. 24,* 1077-1084.
11. Harrison, E.H., and Bowers, W.E. (1983) *J. Biol. Chem. 238,* 7134-7140.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
13. LaBarca, C., and Paigen, K. (1980) *Anal. Biochem. 102,* 344-352.
14. Amar-Costesec, A., Beaufay, H., Wibo, M., Thines-Sempoux, D., Feytmans, E., Robbi, M., and Berthet, J. (1974) *J. Cell Biol. 61,* 201-212.
15. Beaufay, H., Amar-Costesec, A., Thines-Sempoux, D., Wibo, M., Robbi, M., and Berthet, J. (1974) *J. Cell Biol. 61,* 213-231.
16. Amar-Costesec, A., Wibo, M., Thines-Sempoux, D., Beaufay, H., and Berthet, J. (1974) *J. Cell Biol. 62,* 717-745.
17. Reddy, J.K., Azarnoff, D.L., and Sirtori, C.R. (1978) *Arch. Int. Pharmacodyn. 234,* 4-14.
18. Moody, D.E., and Reddy, J.K. (1976) *J. Cell Biol. 71,* 768-780.
19. Reddy, J.K., Rao, M.S., Azarnoff, D.L., and Sell, S. (1979) *Cancer Res. 39,* 152-161.
20. George, M.E., and Andersen, M.E. (1986) *The Toxicologist 6,* 1264.
21. Poland, A., and Kimbrough, R.D. (eds.) (1984) *Biological Mechanisms of Dioxin Action,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
22. Fowler, B.A., Lucier, G.W., Brown, H.W., and McDaniel, O.S. (1973) *Environ. Health Perspect. 5,* 141-146.
23. Jones, G., and Butler, W.H. (1974) *J. Pathol. 112,* 93-97.

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Effects of Octylglucoside and Triton X-100 on the Kinetics and Specificity of Carnitine Palmitoyltransferase

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The effects of octylglucoside on the substrate specificity, kinetics and aggregation state of purified carnitine palmitoyltransferase (CPT) from beef heart mitochondria were investigated and compared to the effects of Triton X-100. Conditions in which CPT can be assayed in the absence of micelles and albumin, thereby eliminating miceller effects on the kinetic parameters, are described. When octylglucoside is substituted for Triton X-100, the specificity of CPT in the forward direction shifts towards the long-chain acyl-CoAs, and large changes in the kinetic constants are observed. The $K_{0.5}$ for L-carnitine varied as much as 50-fold, depending on the acyl-CoA and detergent used. At pH 8.0 and 200 μM palmitoyl-CoA, the $K_{0.5}$ for L-carnitine is 4.9 mM in 12 mM octylglucoside and 0.2 mM in 0.1% Triton X-100. Octylglucoside enhances the activity of CPT with long-chain acyl-CoA and lowers the $K_{0.5}$ for these substrates. At pH 6.0, the $K_{0.5}$ for palmitoyl-CoA is 24.2 μM in 0.1% Triton X-100, in contrast to 3.1 μM in 12 mM octylglucoside. Octylglucoside is a competitive inhibitor of CPT with octanoyl-CoA as substrate with a K_i of 15 mM. Nonlinear kinetics for both acyl-CoAs and L-carnitine are observed when the concentration of octylglucoside is reduced to less than half of its critical micellar concentration (cmc). Gel filtration of CPT in octylglucoside below its cmc gives a single protein peak with a molecular mass of ca. 660,000 daltons. These data indicate that the catalytically active form of purified CPT is an aggregate that has quaternary structure and must have a very flexible catalytic site whose affinity for substrate and catalytic efficiency can be altered greatly by changes in environment and experimental conditions. *Lipids 23,* 120–125 (1988).

The acyl-CoA specificity of carnitine acyltransferases has been used to designate short- (acetyl), medium- (octanoyl) and long- (palmitoyl) chain acyltransferases (1,2). However, the substrate specificity profile of carnitine palmitoyltransferase (CPT) from beef heart mitochondria in the reverse direction (formation of acyl-CoA) is affected by micelles of either substrate or nonionic detergent (3). Therefore, the choice of experimental conditions for the determination of these profiles can greatly influence the results. Undoubtedly, this is a major factor in the large differences in the data from different laboratories reporting on the same enzyme (1). For example, Kopec and Fritz (4) reported relative rates of 2, 31 and 100% for octanoyl-, lauryl-, and palmitoylcarnitine esters using purified calf liver mitochondrial CPT, while West et al. (5) reported relative rates of 93, 152 and 100% for the same substrates using partially purified ox liver CPT. Clarke and Bieber (3) duplicated the different specificity profiles by varying the experimental conditions.

CPT from beef heart mitochondria has a high activity (V_{max}) for medium-chain acyl moieties in the forward direction but it shows a higher activity with long-chain acyl moieties in the reverse direction (6). Such specificity patterns have been attributed to increased substrate inhibition at the L-carnitine site, as the length of the acyl group increases (7,8). It has been proposed that detergents that activate the enzyme, such as Triton X-100 and (+)palmitoylcarnitine (9), prevent the acyl-CoA competitive binding at the L-carnitine site.

Previously, we determined the kinetics of purified beef heart mitochondrial CPT in micellar concentration (0.1%) of Triton X-100 (10). These data showed that the affinity of the enzyme for L-carnitine depends on the acyl-CoA chain length, namely a decrease in the chain-length of the acyl-CoA logarithmically increases the $K_{0.5}$ for L-carnitine. Thus, the concentration of L-carnitine can affect the "physiological substrate specificity" (11) of CPT and high activity with medium-chain acyl-CoA only occurs at high L-carnitine concentrations. The previous studies did not provide information about the effect of different environments, i.e. detergents, on the kinetic behavior of CPT. In these studies, the acyl-CoA specificity, substrate kinetics and aggregation state of CPT in the absence of detergent micelles in 12 mM octylglucoside have been determined.

EXPERIMENTAL PROCEDURES

Enzyme purification and detergent exchange. CPT was purified from beef heart mitochondria as described (10). Cibacron Blue Sepharose chromatography, the final step in the purification, also was used to exchange octylglucoside for Triton X-100 as follows. The concentration of Triton X-100 in the equilibrating buffer for the Cibacron Blue Sepharose column was reduced to 0.002%. After loading the partially purified enzyme, the column was washed with one bed volume of buffer in 0.002% Triton X-100 followed by three to five bed volumes of buffer in 25 mM octylglucoside. The enzyme was eluted by increasing the ionic strength of the buffer in 25 mM octylglucoside as described previously (10). The concentration of octylglucoside in the eluted enzyme was reduced to 12 mM by dialysis.

Determination of micelle formation. The formation of micelles was monitored by addition of either acyl-CoA or octylglucoside to a cuvette containing the assay media and 4 μM pinacyanol chloride. Formation of micelles is observed by a change in the extinction coefficient of the dye at 610 nm (12). This method was chosen over others because it gives the lowest estimates of critical micellar concentrations (cmc) for the acyl-CoAs.

Molecular weight determination. A 50 × 1.5 cm Fractogel TSK HW-55 column was equilibrated with buffer

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Abbreviations: Bis Tris Propane, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; cmc, critical micellar concentration; CPT, carnitine palmitoyltransferase; DTBP, 4, 4′-dithiobis-pyridine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

containing 2.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 0.25 mM ethylenediamine tetraacetic acid (EDTA), 300 MM KCl and 12 mM octylglucoside at pH 8.0. The column was calibrated using 10 mg/ml each of ovalbumin, BSA, aldolase, thyroglobulin and 1 mg/ml of ferritin. One unit of purified CPT was loaded, and 0.3 ml fractions were collected.

Assay methods. CPT activity was measured with the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) continuous rate assay as described (13). Kinetic measurements with purified CPT were made as described (10). The 2.0 ml reaction volume contained 50 mM 1,3-bis[tris(hydroxylmethyl)-methylamino]propane (Bis Tris Propane) buffer pH 6 or 8, 1.1 mM EDTA, 150 μM DTNB for pH 8 or 150 μM 4,4′-dithiobis-pyridine (DTBP) for pH less than 8 and concentrations of octylglucoside or 0.1% Triton X-100, L-carnitine and acyl-CoA as indicated in the figures and tables at 25 C, with (0.05–0.1) μg/ml of enzyme. Protein was determined by a fluorescamine method (14).

RESULTS

Effect of octylglucoside on CPT activity. Optimum CPT activity was obtained with 12 mM octylglucoside when palmitoyl-CoA was the substrate but the response with octanoyl-CoA was very different (Fig. 1). Increasing concentrations of octylglucoside above 12 mM had little effect on the activity of CPT with octanoyl-CoA but strong inhibition of activity occurred with palmitoyl-CoA as substrates.

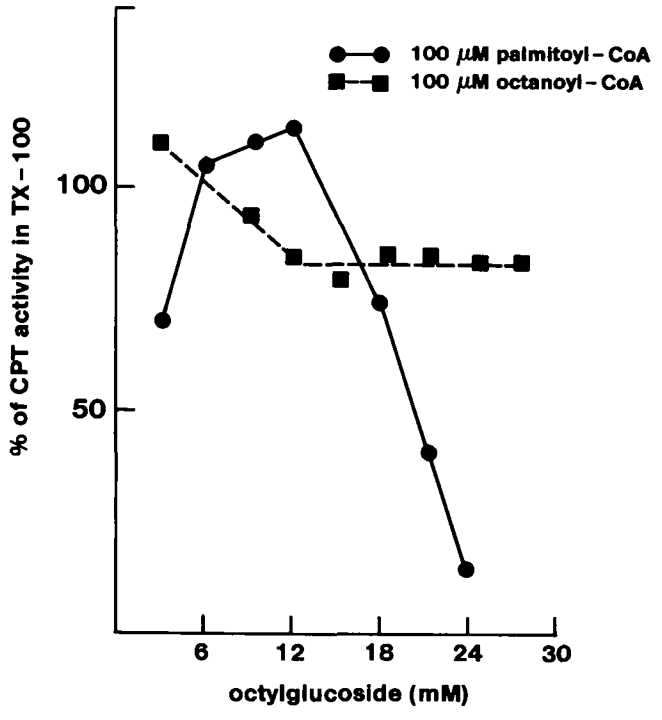

FIG. 1. Effect of octylglucoside on CPT activity. Activity was measured in the forward direction with the DTNB assay. L-carnitine was 1.1 mM and the acyl-CoA was 100 μM. Velocities are plotted relative to the V_{max} in 0.1% Triton X-100. The V_{max} in 0.1% Triton X-100 was 42 units/mg protein.

Substrate specificity profile of CPT in 12 mM octylglucoside and in Triton X-100. When octylglucoside is substituted for Triton X-100, changes in the acyl-CoA specificity pattern of CPT are apparent. For the shaded areas in Figure 2A and 2B, the patterns were determined with 1.1 mM L-carnitine and 200 μM acyl-CoAs. Although maximum activity with 1.1 mM carnitine was obtained with decanoyl-CoA in both detergents, the relative velocity with octanoyl-CoA was much less in octylglucoside. The acyl-CoA specificity with saturating levels of L-carnitine also is shown in Figure 2A and 2B (unshaded areas). In Triton X-100, the enzyme has the highest V_{max} with hexanoyl-CoA, and the V_{max} decreases as the acyl-CoA chain-length increases. In octylglucoside, the velocity is greatest with decanoyl-CoA.

Determination of nonmicellar assay conditions. To select suitable conditions in which to assay the enzyme in the absence of detergent micelles or mixed substrate/detergent micelles, the dye pinacyanol chloride was used to detect formation of micelles in the assay media. The cmc of octylglucoside in the assay media is greater than 30 mM, and in the absence of detergent palmitoyl-CoA forms micelles at 5.4 μM (data not shown). With 12 mM octylglucoside, no detergent micelles were detected but addition of palmitoyl-CoA to the assay media containing 12 mM octylglucoside reduced the cmc of palmitoyl-CoA to about 1 μM (Fig. 3). Octanoyl-CoA has a cmc greater than 200 μM in the assay media in the absence of detergent (data not shown). Addition of up to 150 μM octanoyl-CoA to the assay media containing 12 mM octylglucoside did not cause micelle formation (Fig. 3). Since octanoyl-CoA had a high V_{max} and did not form micelles with the assay conditions, it was used as the substrate for kinetic studies in a nonmicellar environment.

Determination of the molecular weight of CPT in the absence of detergent micelles. The molecular weight of purified CPT was determined in the presence of octylglucoside below its cmc by gel filtration. CPT activity and all detectable protein migrated as a single peak of constant specific activity with a molecular weight of 660,000 daltons (Fig. 4).

Effect of octylglucoside on the kinetics of CPT with L-carnitine. As shown in Table 1, at pH 8.0, the $K_{0.5}$ for L-carnitine is 4.9 mM in 12 mM octylglucoside with 200 μM palmitoyl-CoA. This is in contrast to assays in Triton X-100 in which the $K_{0.5}$ is much lower (0.2 mM). The $K_{0.5}$ for L-carnitine varies slightly with the concentration of the acyl-CoA used as cosubstrate (2.2 mM with 100 μM palmitoyl-CoA and 4.9 mM with 200 μM palmitoyl-CoA; compare values in Tables 1 and 2). Table 1 also shows that with octylglucoside the acyl-CoA chain length does not have the same effect on the $K_{0.5}$ for L-carnitine as in Triton X-100. The $K_{0.5}$ for L-carnitine increases with increasing concentrations of octylglucoside, reaching an apparent maximum of 8.5 mM with octanoyl-CoA and 4.4 mM with palmitoyl-CoA (see Table 2). Data from a typical experiment in nonmicellar assay conditions (12 mM octylglucoside and 100 μM octanoyl-CoA) in which L-carnitine is the varied substrate are shown in Figure 5. The Hill plot gave a n = 1.4. The $K_{0.5}$ for L-carnitine (palmitoyl-CoA as cosubstrate) increases as the pH decreases from 2.2 mM at pH 8.0 to 9.8 mM at pH 6.25 as shown in Table 3. This pH effect was the same as obtained in Triton X-100 (10). The highest V_{max} is at pH 7.0.

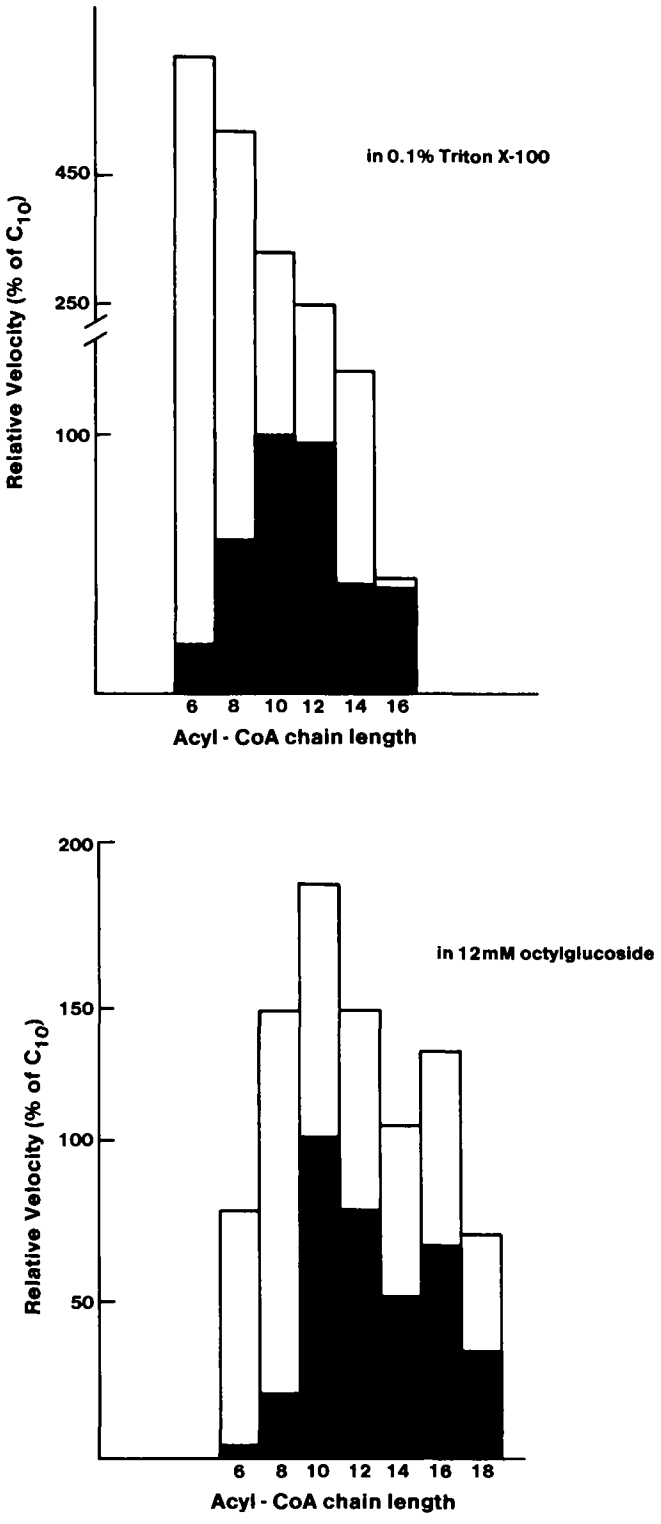

FIG. 2. Comparison of the substrate specificity profiles of CPT in 0.1% Triton X-100 and in 12 mM octylglucoside. For shaded areas, activity was measured with 1.1 mM L-carnitine and 200 μM acyl-CoA, with the kinetic analyzer as described (10). The specific activity of CPT with palmitoyl-CoA in 0.1% Triton X-100 and in 12 mM octylglucoside was 40 and 45, respectively, using saturating amounts of L-carnitine for each acyl-CoA substrate. Since the $K_{0.5}$ for carnitine depends on the acyl-chain length, the optimum L-carnitine level was determined for each. It varied between 6 and 30 mM, depending on the acyl-CoA used (unshaded area).

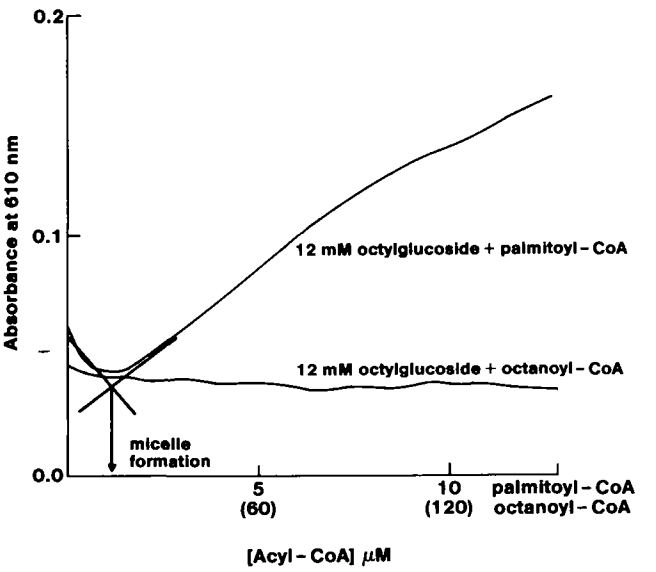

FIG. 3. Determination of the cmc of acyl-CoA in octylglucoside. Pinacyanol chloride (4 μM) was added to the DTNB assay media. The absorbance at 610 was recorded, while palmitoyl-CoA or octanoyl-CoA continuously was added from a pump driven syringe.

TABLE 1

Comparison of the $K_{0.5}$ for L-Carnitine in 12 mM Octylglucoside and 0.1% Triton X-100

Acyl-CoA chain length	Octylglucoside $K_{0.5}$ (mM)	Triton X-100* $K_{0.5}$ (mM)
6	11.1	2.9
8	10.2	1.5
10	11.1	0.64
12	4.2	0.53
14	4.9	0.35
16	4.9	0.2
18	3.8	

200 μM acyl-CoAs at pH 8.0 were used. The kinetic parameters were determined as described (10).

*Data taken from (11), Table 1.

Effect of octylglucoside on kinetic constants for acyl-CoA. Due to the high $K_{0.5}$ for L-carnitine in octylglucoside, its concentration was fixed at 40 mM to saturate the enzyme. As shown in Table 2, increasing concentrations of octylglucoside lowers the $K_{0.5}$ for palmitoyl-CoA. At pH 6.25, the $K_{0.5}$ for palmitoyl-CoA is 24.2 μM in 0.1% Triton X-100 (10) and 3.1 μM in 12 mM octylglucoside. Octylglucoside inhibits CPT activity with octanoyl-CoA and increases the $K_{0.5}$, apparently acting as a weak competitive inhibitor. The apparent K_i for octylglucoside is 15 mM (Fig. 6).

DISCUSSION

Small changes in detergent concentrations caused large changes in some of the kinetic parameters of CPT for medium-chain vs longer-chain acyl-CoAs. This is

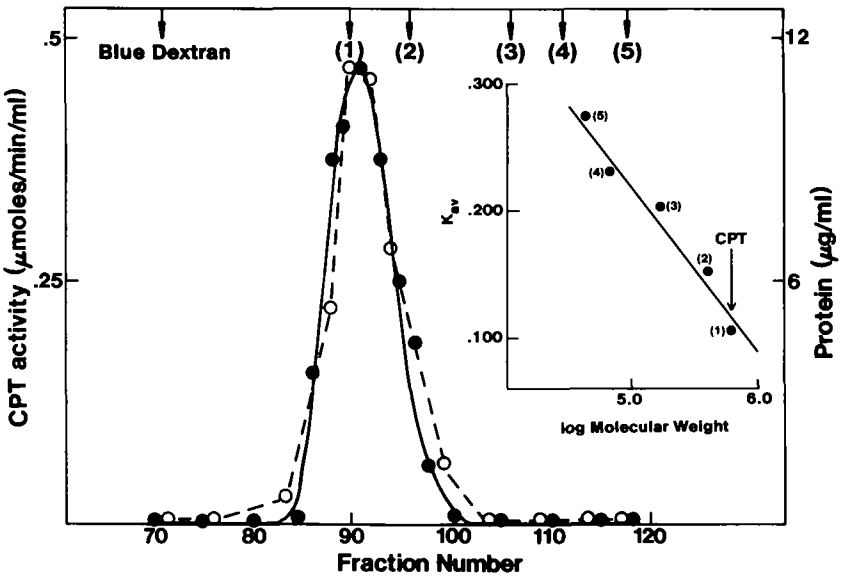

FIG. 4. Determination of the molecular weight of CPT in the absence of detergent micelles. Fractogel TSK HW-55 column was equilibrated with 12 mM octylglucoside as described in experimental procedures. Molecular weight standards were (1) thyroglobulin, (2) ferritin, (3) aldolase, (4) BSA and (5) ovalbumin. Open circles, protein and the closed circles, CPT activity.

TABLE 2

Effect of Octylglucoside Concentration on the $K_{0.5}$ and V_{max} of CPT

Varied substrate	L-Carnitine*				Palmitoyl-CoA**	
Cosubstrate	Octanoyl-CoA (100 μM)		Palmitoyl-CoA (100 μM)		L-Carnitine (40 mM)	
Octylglucoside (mM)	$K_{0.5}$ (mM)	Relative velocity	$K_{0.5}$ (mM)	Relative velocity	$K_{0.5}$ (μM)	Relative velocity
0.1	3.2	1.3	1.4	0.4	10.8	0.7
6	4.2	1.1	1.8	0.7	2.6	0.7
12	9.1	1.0	2.2	1.0	3.1	1.0
18	8.1	1.8	3.1	0.8	3.1	0.8
24	8.2	1.8	4.3	0.8		
30	8.3	2.0	4.5	0.6		

The kinetic parameters were determined as described (10) using the DTBP assay.

*The pH was 8.0.

**The pH was 6.25.

surprising and difficult to interpret. Both the concentration of detergent and the detergent composition affected the substrate specificity pattern of CPT. These data indicate that some of the differences observed in the specificity pattern of CPT for different CPT preparations and different assay conditions are due to the differences in detergent concentrations, cosubstrate concentrations and the type of detergent used. For example, with 12 mM octylglucoside the $K_{0.5}$ for L-carnitine with saturating amounts of medium-chain acyl-CoAs is about 11 mM. This large increase in the $K_{0.5}$ for L-carnitine produced low activities with medium-chain acyl-CoAs when the assay conditions of Figure 1 were used because L-carnitine was 1.1 mM. However, saturation with L-carnitine did not shift the V_{max} of the enzyme towards the 6- and 8-carbon chain acyl-CoA, as occurred in Triton X-100 (Fig. 2). The response of CPT to small changes in octylglucoside concentrations when octanoyl-CoA and palmitoyl-CoA are used as substrates must mean that the enzyme can respond to subtle changes in its environment. Increasing the concentration of octylglucoside from 12 to 24 mM had no effect on the rate with octanoyl-CoA but it inhibited the rate with palmitoyl-CoA ca. 80%. Such data suggest that perturbations of the active site may have more of an effect on catalysis when long-chain acyl-CoAs are the substrate or that some type of inhibitory palmitoyl-CoA/octylglucoside mixed micelle is formed whose concentration is dependent on the detergent concentration. Alternately, the effect of detergent on the velocity could be at the presumed alternate acyl-CoA

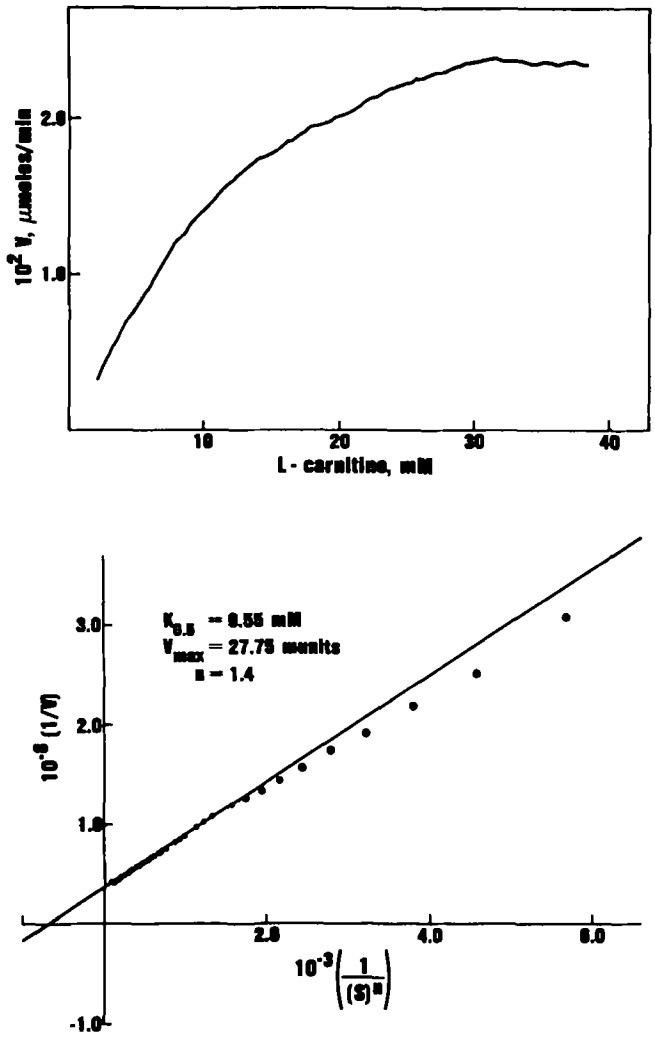

FIG. 5. Effect of L-carnitine concentration on CPT velocity. The concentration of octanoyl-CoA was 100 μM and L-carnitine was varied at pH 8.0. A double-reciprocal plot (1/v vs $1/s^n$) with a Hill n = 1.4 is shown in the bottom.

binding site that is involved in the acyl-CoA induced positive substrate cooperativity. The constancy of the Hill n seems to preclude the latter suggestion.

The changes in kinetic behavior of CPT caused by changes in experimental conditions are consistent with purified CPT being an oligomeric enzyme. The activities of such enzymes often are very responsive to small changes in their environment. The purified enzyme in octylglucoside is an oligomer. Although it has a monomeric molecular weight of 67,000 (3), its molecular weight in the absence of detergent micelles is about 660,000 (Fig. 4). Previously, we tentatively concluded CPT exists as an aggregate of about four to six monomers in 0.1% Triton X-100 (10). Our data now show that the aggregated molecular weight for the purified enzyme reported previously is not caused by several enzyme monomers binding to a detergent micelle. The slightly higher molecular weight estimate in 12 mM octylglucoside could indicate a higher aggregate, i.e. eight monomers, in the absence of detergent micelles or, alternatively, a greater amount of detergent bound to the enzyme. The amount of detergent bound to CPT is not known. The kinetics of the enzyme indicate that there is considerable interaction between the

TABLE 3

Effect of pH on the Kinetic Parameters of CPT for L-Carnitine in Octylglucoside

pH	$K_{0.5}$ mM	V_{max} milliunits	Hill n
6.25	9.8	14.8	1.5
6.5	7.4	14.7	1.5
6.75	5.0	13.3	1.6
7.0	4.6	17.8	1.5
7.25	3.9	12.5	1.5
7.5	3.0	12.0	1.5
8.0	2.2	10.1	1.5

Palmitoyl-CoA was 100 μM. Octylglucoside was 12 mM.
The kinetic parameters were determined as described (10) using the DTBP assay.

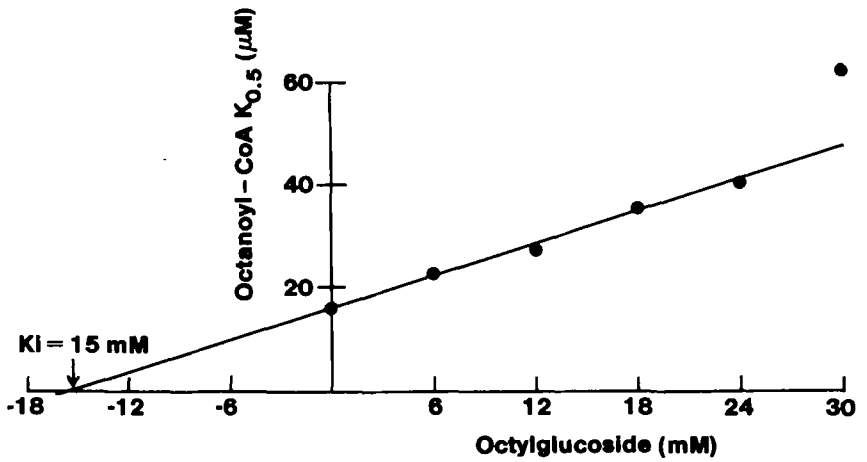

FIG. 6. Effect of octylglucoside on the $K_{0.5}$ for octanoyl-CoA. The $K_{0.5}$'s were determined as described (10) using the DTBP assay at pH 6.0. L-carnitine was 40 mM.

monomers, since the Hill coefficients for both substrates are greater than 1.0. The molecular weight of native CPT is unknown but the sigmoid kinetics for both cosubstrates with the membrane-bound enzyme (unpublished data) suggest interaction among subunits also occurs in the membrane-bound state. It has been shown that CPT of rat liver mitochondria also is an oligomer but its aggregated molecular weight is about half that reported herein for the enzyme from beef heart (16).

The data are similar to that observed in Triton X-100 in the following: the enzyme's kinetic parameters are strongly influenced by pH, the purified enzyme does not show inhibition by malonyl-CoA and it shows sigmoidal kinetics with both substrates even in the absence of detergent micelles. However, octylglucoside increases the $K_{0.5}$ for L-carnitine up to 25-fold compared with the $K_{0.5}$ in Triton X-100 and the relationship between the acyl-CoA chain length and the $K_{0.5}$ for L-carnitine is very different. With medium-chain acyl-CoA, the $K_{0.5}$ for L-carnitine is about twice the $K_{0.5}$ for L-carnitine with long-chain acyl-CoA (Table 2), and the large decrease in the $K_{0.5}$ for L-carnitine as the acyl chain lengthens is not observed (Table 1). The data do not differentiate between octylglucoside binding at an allosteric site and acting as a weak negative effector or its binding at the carnitine binding site, thereby having a direct effect on the $K_{0.5}$ for L-carnitine. The large increase in $K_{0.5}$ for L-carnitine in the presence of octylglucoside indicates a very flexible substrate binding site that responds to its environment.

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REFERENCES

1. Hoppel, C.L. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A., ed.) Vol. 2, pp. 119-143, Plenum Press, New York, NY.
2. Bieber, L.L., and Farrell, S.O. (1983) *Enzymes 16,* 627-644.
3. Clarke, P.R.H., and Bieber, L.L. (1981) *J. Biol. Chem. 256,* 9869-9873.
4. Kopec, B., and Fritz, I.B. (1971) *Can. J. Biochem. 49,* 941-948.
5. West, D.W., Chase, J.F.A., and Tubbs, P.K. (1971) *Biochem. Biophys. Res. Commun. 42,* 912-918.
6. Clarke, P.R.H., and Bieber, L.L. (1981) *J. Biol. Chem. 256,* 9861-9868.
7. Bremer, J., and Norum, K.R. (1967) *J. Biol. Chem. 242,* 1744-1748.
8. Bremer, J. (1983) *Physiol. Rev. 63,* 1420-1480.
9. Bremer, J., and Norum, K.R. (1967) *J. Biol. Chem. 242,* 1749-1755.
10. Fiol, C.J., and Bieber, L.L. (1984) *J. Biol. Chem. 259,* 13084-13088.
11. Fiol, C.J., and Bieber, L.L. (1984) *Curr. Top. Cell. Regul. 24,* 111-117.
12. Tippett, P.S., and Neet, K.E. (1982) *J. Biol. Chem. 257,* 12839-12845.
13. Bieber, L.L., Abraham, T., and Helmrath, T.V. (1972) *Anal. Biochem. 50,* 509-518.
14. Bohlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) *Arch. Biochem. Biophys. 155,* 213-220.
15. Clarke, P.R.H., and Bieber, L.L. (1981) *J. Biol. Chem. 256,* 9859-9873.
16. Miyozawa, S., Ozasa, H., Osumi, T., and Hashimota, T. (1983) *J. Biochem. 94,* 529-542.

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Cupric Ion-dependent Inhibition of Lysosomal Acid Cholesteryl Ester Hydrolase in the Presence of Hydroxylamine

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In the presence of hydroxylamine or ascorbic acid, the inhibitory effects of Cu^{2+} on lysosomal acid cholesteryl ester hydrolase (acid CEH) partially purified from rat liver were studied.

Hydroxylamine stimulated the inhibition of acid CEH activity by Cu^{2+} but not that by Zn^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+} and Hg^{2+}. This Cu^{2+}-dependent inhibition of acid cholesterol ester hydrolase (CEH) activity was completely prevented by ethylenediamine tetraacetic acid (EDTA), EGTA and o-phenanthroline, a chelator with a stability constant for Cu^{2+}, and also by sulfhydryl agents and cytoplasmic reducing agents such as cysteine, glutathione and mercaptoethanol. In addition, the stimulative effects of hydroxylamine on Cu^{2+}-dependent inhibition were maintained even after preincubation of Cu^{2+} with hydroxylamine.

On the other hand, ascorbic acid was found to replace the stimulation by hydroxylamine of the Cu^{2+}-dependent inhibition of acid CEH activity but the effects of ascorbic acid progressively became smaller with prolongation of the preincubation time. Moreover, addition of chemical radical scavengers to the reaction mixture did not prevent the Cu^{2+}-dependent inhibition of acid CEH activity in the presence of ascorbic acid. These results suggest that Cu^{2+} causes inhibition of lysosomal acid CEH activity through the formation of Cu^{1+} in a reductive medium. *Lipids 23,* **126–130 (1988).**

Acid cholesterol ester hydrolase (CEH), which is found in several tissues including those of the liver, spleen and aorta, is distributed broadly in all subcellular fractions although the major proportion of its activity appears to be localized in the lysosomal fraction (1–7). It has been suggested that acid CEH may play a role in the catabolism of cellular cholesteryl esters (8). We previously have reported that a cytosolic protein in rat liver inhibits acid CEH activity (7). However, the mechanism involved in the regulation of lysosomal acid CEH is not known.

On the other hand, hypercholesterolemia in cupric ion-deficient rats has been observed by many investigators (9–12), and we also have shown that the acid CEH activity in rats treated with cupric ion is decreased markedly in comparison with that in normal rats (13). However, the role of cupric ion in cholesterol metabolism still is unknown. In this study, on the basis of the above observations concerning the effect of cupric ion on serum and liver cholesterol metabolism, we tried to clarify the mechanism of Cu^{2+}-dependent inhibition of acid CEH activity in the presence of reducing agents such as ascorbic acid or hydroxylamine in vitro.

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Abbreviations: CEH, cholesterol ester hydrolase; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid.

MATERIALS AND METHODS

Chemicals and radiochemicals. Cholesteryl [1-^{14}C] oleate (sp. act. 58.6 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Phenolphthalein glucuronide, phenylphosphate, hemoglobin, cysteine, glutathione, sodium thiocyanate, mannitol, bilirubin, guanosine and catalase were purchased from Sigma Chemical Co. (St. Louis, MO). L-ascorbic acid, hydroxylamine and 2,2-biquinoline were purchased from Tokyo Kasei Chemical Co., Ltd. (Japan). Hydroxyapatite was purchased from Seikagaku Kogyo Co., Ltd. (Japan). Phenyl Sepharose CL-4B, Polybuffer Exchanger 94 and Polybuffer 74 were obtained from Pharmacia Fine Chemicals (Sweden).

Acid cholesteryl ester hydrolase assay. The activity of acid CEH was measured by the method of Brecher et al. (14). Preparation of the substrate was performed as described (7). This sensitive microassay was carried out in 0.1 M acetate buffer (pH 5.0) in a final assay volume of 0.3 ml. All assays were done using quantities of protein (usually less than 1 μg) and incubation times (30 min) that ensured nearly linear rates of substrate hydrolysis. All data points reported here represent the means of triplicate assays.

Assay of lysosomal marker enzymes. Cathepsin D was assayed by the method of Hirado et al. (15), and the amount of reaction products was assayed by the method of Lowry et al. (16). β-Glucuronidase was assayed using phenolphthalein glucuronide as the substrate. Phenolphthalein liberated from the substrate was measured by the method of Gianetto and De Duve (17). Acid phosphatase was measured using phenylphosphate as the substrate, essentially as described (18). Protein concentration was assayed by the method of Lowry et al. (16).

Determination of Cu^{1+} content. The determination of Cu^{1+} content in the reaction mixture was performed by the colorimetric procedure described by Sandell (19).

Purification of acid cholesteryl ester hydrolase. All preparation steps were carried out at 4 C. Acid CEH was isolated by a modification of the method of Klemets and Lundberg (20). Normal rat liver was homogenized in four volumes of ice-cold 0.25 M sucrose/1 mM ethylenediamine tetraacetic acid (EDTA)/0.01 M Tris-HCl buffer (pH 7.5). For the preparation of lysosomal fractions from liver, the method of Brecher et al. was followd (14). The precipitate (lysosome-rich fraction) was suspended in 0.2% Triton X-100/0.01 M sodium phosphate buffer (pH 7.0). The suspension was stirred at 4 C for 30 min and centrifuged at 50,000 × g for 30 min. The supernatant was applied to a hydroxyapatite column (5 × 10 cm) previously equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) containing 5 mM mercaptoethanol. After washing the column with the same equilibration buffer, the enzyme was eluted with 0.05 M sodium phosphate buffer (pH 7.0). The enzyme fractions were pooled. The pooled fractions were applied to a column of Phenyl Sepharose CL-4B (2 × 10 cm) equilibrated with 0.01 M sodium phosphate

buffer (pH 7.0) containing 5 mM mercaptoethanol. After washing the column with the same equilibration buffer, elution was performed with a linear concentration gradient of 0 to 90% ethylene glycol in the same buffer. Fractions with enzyme activity were pooled and dialyzed against 5 l of 0.01 M sodium phosphate buffer (pH 7.0) containing 5 mM mercaptoethanol. The dialyzed fraction was applied to a column of Polybuffer Exchanger 94 (2 × 10 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). The column was washed thoroughly with the same buffer, and then the enzyme was eluted with Polybuffer 74 at a pH gradient from 7.0 to 4.0. The fractions containing the highest enzyme activity were pooled, concentrated by membrane filtration and applied to a column of Sephadex G-75 to remove Polybuffer 74. The resulting partially purified protein preparation showed one major band and a few weak bands when examined by SDS-polyacrylamide gel electrophoresis according to Laemmli (21), the molecular weight of this major band was about 59,000 daltons (20). The pH optimum of the partially purified acid CEH was 5.0, and the enzyme had a specific activity of 30 μmol per min per mg protein. The preparation was stored at −30 C and used in all experiments.

RESULTS

Acid CEH partially purified from rat liver lysosomes was incubated with increasing concentrations of Cu^{2+} in the presence or absence of hydroxylamine. As shown in Figure 1, hydroxylamine markedly stimulated the inhibitory effect of Cu^{2+} on acid CEH activity, and the degree of the stimulatory effect was dependent upon the concentration of Cu^{2+}, the addition of about 8 μM Cu^{2+} causing approximately half-maximal inhibition of acid CEH activity. However, in the absence of hydroxylamine, Cu^{2+} showed an inhibitory effect only at high concentrations.

Figure 2 shows the effects of hydroxylamine or ascorbic acid concentration on the inhibition of acid CEH activity with or without Cu^{2+}. Acid CEH activity was inhibited significantly at a concentration of 50 μM of either hydroxylamine or ascorbic acid in the presence of Cu^{2+} (15 μM) but hydroxylamine and ascorbic acid did not by themselves inhibit acid CEH activity, even at higher concentrations. Therefore, we examined the effect of various radical scavengers on the Cu^{2+}-dependent inhibition of acid CEH activity in the presence of ascorbic acid. As shown in Table 1, these scavengers did not prevent the Cu^{2+}-dependent inhibition of acid CEH activity in the presence of ascorbic acid except for sodium thiocyanate.

It is well known that acid CEH is inhibited by certain divalent cations (4,7,22,23). Therefore, the stimulative effects of hydroxylamine on the inhibition of acid CEH activity by these divalent metal ions were compared (Table 2). Fe^{2+} (0.05 mM), Hg^{2+} (0.5 mM) and Zn^{2+} (1.0 mM) inhibited acid CEH activity up to about 62%, 49% and 60%, respectively, in the absence of hydroxylamine but

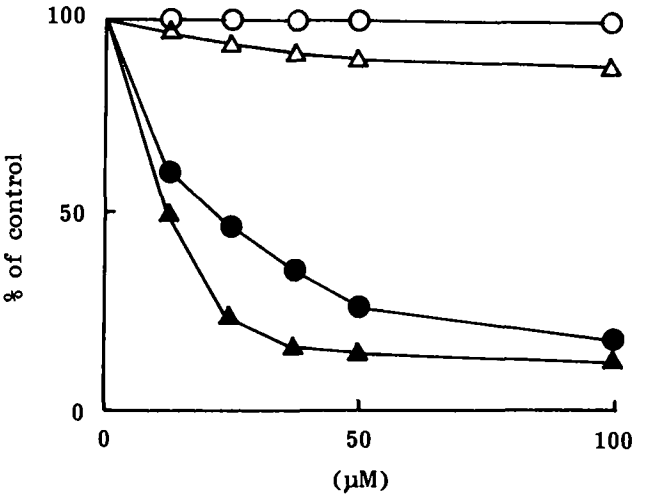

FIG. 2. Effect of hydroxylamine (●, ○) or ascorbic acid (▲, △) on acid CEH activity in the presence (closed symbols) and absence (open) of 15 μM Cu^{2+}. The 100% value is the activity under standard assay conditions.

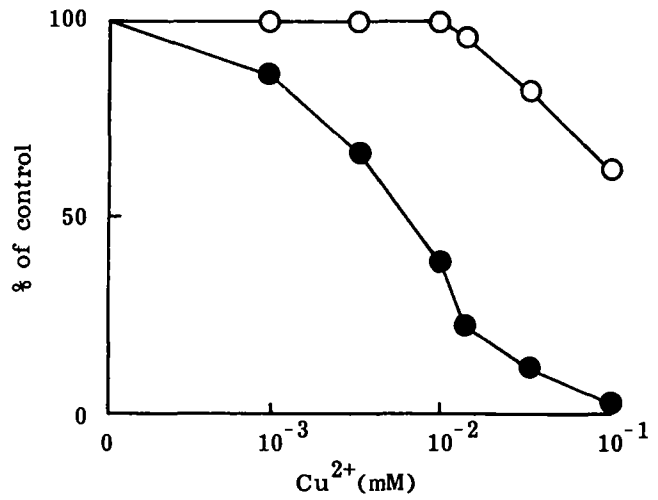

FIG. 1. Inhibition by Cu^{2+} of acid CEH activity in the presence (●) and absence (○) of 50 μM hydroxylamine. The 100% value is the activity under standard assay conditions.

TABLE 1

Effect of Radical Scavengers on Cu^{2+}-dependent Inhibition of Acid CEH Activity in the Presence of Ascorbic Acid

Scavenger	Final concn. (mM)	Activity (%)
None	—	18.7
Mannitol	1.0	16.8
Ethanol	1.0	18.9
Sodium benzoate	1.0	19.2
Potassium iodide	1.0	10.4
Guanosine	0.1	13.9
Bilirubin	0.5	10.7
Sodium thiocyanate	1.0	45.9
DMSO	1.0	19.4
Catalase	50 μg/ml	18.3

Reaction mixture contained ascorbic acid (50 μM), Cu^{2+} (20 μM) and the specified amounts of various scavengers in 0.1 M acetate buffer (pH 5.0). The 100% value was the activity under standard assay conditions. Activity was determined as described in the Materials and Methods section.

none of these divalent cations produced any increment in the inhibition of acid CEH activity like that observed when Cu^{2+} and hydroxylamine were coexistent.

Next, we tested the effects of chelators, sulfhydryl agents and reducing agents on the Cu^{2+}-dependent inhibition of acid CEH activity by hydroxylamine. As shown in Table 3, the Cu^{2+}-dependent inhibition of acid CEH activity in the presence of hydroxylamine was effectively prevented when 125 μM EDTA was present in the incubation medium. At the same concentrations, EGTA and o-phenanthroline also were able to replace EDTA and produce a preventive effect. Moreover, a similar effect was brought about by the sulfhydryl and reducing agents such as cysteine, glutathione and mercaptoethanol at the same concentrations (Table 3).

On the other hand, the inhibitory effect of Cu^{2+} was tested on the activity of other lysosomal enzymes, cathepsin D, acid phosphatase and β-glucuronidase in the presence or absence of hydroxylamine. The activities of these enzymes were not changed in the presence of Cu^{2+} and hydroxylamine (data not shown).

For further investigation of the enhancement by hydroxylamine of Cu^{2+}-dependent inhibitory action, the effects of preincubation of Cu^{2+} with hydroxylamine or ascorbic acid on acid CEH activity were studied (Fig. 3a, 3b). Even when Cu^{2+} was preincubated with hydroxylamine for 30 min, the level of Cu^{2+}-dependent inhibition of acid CEH activity did not change. However, in the case of ascorbic acid the Cu^{2+}-dependent inhibitory effects on acid CEH activity progressively became smaller with

TABLE 2

Effect of Divalent Metals on Acid CEH Activity in the Presence or Absence of Hydroxylamine

Divalent metal	Final concn. (mM)	Activity (%)	
		0 μM	50 μM
		Hydroxylamine	
None	—	100.0	100.0
Cu^{2+}	0.02	90.6	20.3
Fe^{2+}	0.05	61.5	49.5
Hg^{2+}	0.5	48.7	54.3
Zn^{2+}	1.0	60.4	65.8
Co^{2+}	2.0	89.6	92.5
Mn^{2+}	2.0	80.4	86.4
Ca^{2+}	2.0	86.5	85.8
Mg^{2+}	2.0	90.6	88.3

All agents were solubilized in water. The 100% value was the activity under standard assay conditions. Activity was determined as described in the Materials and Methods section.

TABLE 3

Effect of Chelating, Reducing and Sulfhydryl Agents on Cu^{2+}-Dependent Inhibition of Acid CEH Activity in the Presence of Hydroxylamine

Agent	Final concn. (μM)	Activity (%)
None	—	20.5
EDTA	125	98.6
	62	90.5
EGTA	125	96.5
	62	91.6
o-Phenanthroline	250	98.3
	125	90.2
Cysteine	500	90.5
	250	89.1
Glutathione	250	95.7
	125	90.1
Mercaptoethanol	250	90.8
	125	89.1

Reaction mixture contained hydroxylamine (50 μM), Cu^{2+} (20 μM), and the specified amounts of various agents in 0.1 M acetate buffer (pH 5.0). The 100% value was the activity under standard assay conditions. Activity was determined as described in the Materials and Methods section.

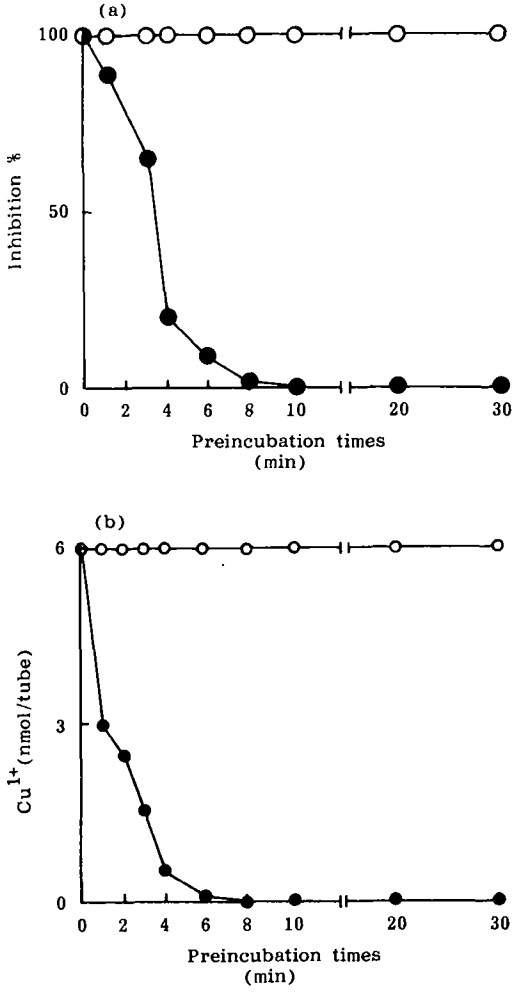

FIG. 3. Effect of the preincubation times on acid CEH activity (a) and Cu^{1+} contents (b) in the presence of Cu^{2+}-ascorbic acid (●) or Cu^{2+}-hydroxylamine (○). The preincubation medium contained 30 μM $^{2+}$ and 50 μM ascorbic acid or 50 μM hydroxylamine. After preincubation, (a) enzyme was incubated with the preincubation medium in 0.1 M acetate buffer (pH 5.0) in a final assay volume of 0.3 ml, (b) Cu^{1+} content was determined as described under Materials and Methods.

prolongation of the preincubation time, and the effects disappeared completely after preincubation for 8–10 min.

On the other hand, the amount of Cu^{1+} formed during preincubation with Cu^{2+} in the presence of hydroxylamine or ascorbic acid was measured by the Cu^{1+}-2,2-biquinoline complex method. As shown in Figure 3b, when Cu^{2+} was preincubated with hydroxylamine the amount of Cu^{1+} detected after a further 30-min period almost was identical to that measured after a two-min preincubation. In contrast, the amount of Cu^{1+} formed in the presence of ascorbic acid gradually decreased in parallel with the disappearance of Cu^{2+}-dependent inhibition.

Since Cu^{1+} is thought to be a powerful inhibitor of the acid CEH, we examined the direct effect of Cu^{1+} on acid CEH activity. Acid CEH was inhibited significantly by Cu^{1+}, the addition of about 10 μM Cu^{1+} causing approximately half-maximal inhibition of acid CEH activity (Fig. 4).

DISCUSSION

The results presented here show that hydroxylamine has a stimulative effect on Cu^{2+}-dependent inhibition of lysosomal acid CEH activity in rat liver. This inhibitory effect was shown to be dependent upon the Cu^{2+} concentration, and hydroxylamine did not inhibit the enzyme activity. Furthermore, this stimulative effect of hydroxylamine was decreased by the addition of chelating agents or sulfhydryl compounds. In addition, when Cu^{2+} was replaced by other divalent cations such as Fe^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+} and Hg^{2+}, the stimulative effects of hydroxylamine were not observed. These results suggest that the stimulation by hydroxylamine of the Cu^{2+}-dependent inhibitory effect on acid CEH activity may be a specific characteristic of Cu^{2+}.

On the other hand, the sensitivity of ascorbic acid to the catalytic effects of minute traces of cupric ion is well known. In addition, transition metal ions such as Cu^{2+} and Fe^{2+} are known to exert a catalytic effect upon the oxidation of ascorbic acid. When hydroxylamine was replaced by ascorbic acid, the latter also revealed a similar inhibition of acid CEH activity in the presence of Cu^{2+}. However, as the preincubation time of Cu^{2+} with ascorbic acid was prolonged, Cu^{2+}-dependent inhibition resulted in a progressively smaller effect. These results suggested that an oxidized form of ascorbic acid was much less effective for inhibition of the enzyme. It is well known that activated oxygen species such as hydroxyl radicals and superoxide radicals are formed in the reaction of ascorbic acid and Cu^{2+} (24). Various radical scavengers, therefore, were added to the incubation medium containing acid CEH, and none of them prevented the Cu^{2+}-dependent inhibition in the presence of ascorbic acid with the exception of sodium thiocyanate. The slight recovery of Cu^{2+}-dependent inhibition in addition of sodium thiocyanate appears to be dependent upon the formation of insoluble cuprous thiocyanate. These results suggest that the inhibition of acid CEH activity by ascorbic acid and Cu^{2+} may not be due to activated oxygen species.

On the other hand, it also is known that Cu^{1+} is formed in the reaction of ascorbic acid or hydroxylamine with Cu^{2+} (19,25–27). When Cu^{2+} was preincubated with hydroxylamine, the degree of Cu^{2+}-dependent inhibition of acid CEH activity was maintained at the same level, and moreover, the amount of Cu^{1+} in the reaction mixture did not change at all during the preincubation period. However, in the combination of Cu^{2+} and ascorbic acid, neither Cu^{2+}-dependent inhibition nor Cu^{1+} in the reaction mixture could be detected for a few minutes. Moreover, the direct addition of Cu^{1+} to the incubation medium inhibited acid CEH activity at the similar levels to that obtained under coexistence with Cu^{2+} and ascorbic acid or hydroxylamine. These results indicated that Cu^{2+}-dependent inhibition of acid CEH activity in the presence of hydroxylamine or ascorbic acid was specifically caused by the Cu^{1+} formed in a reductive medium.

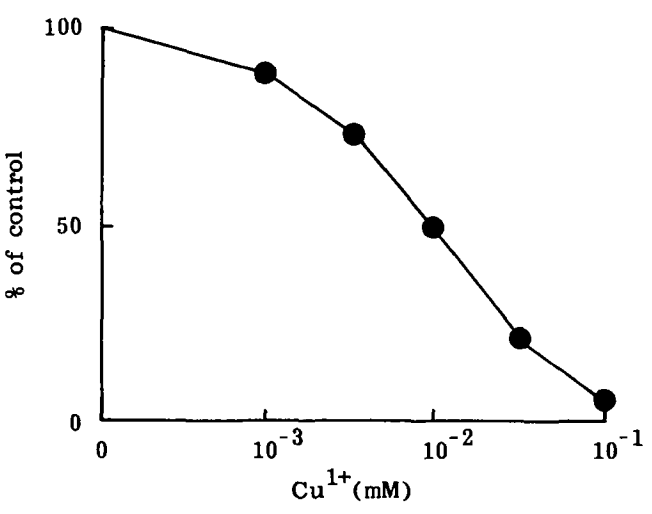

FIG. 4. Inhibition by Cu^{1+} of acid CEH activity. CuCl was solubilized in DMSO. The 100% value is the activity under standard assay conditions.

REFERENCES

1. Stokke, K.T. (1972) *Biochim. Biophys. Acta 280,* 329–335.
2. Nilsson, A., Norden, H., and Wilhelmsson, L. (1973) *Biochim. Biophys. Acta 296,* 593–603.
3. Takano, T., Black, W.J., and De Duve, C. (1974) *J. Biol. Chem. 249,* 6732–6737.
4. Brown, W.J., and Sgoutas, D.S. (1980) *Biochim. Biophys. Acta 617,* 305–317.
5. Dousset, J.C., Dousset, N., Foglietti, M.J., and Douste-Blazy, L. (1981) *Biochim. Biophys. Acta 664,* 273–277.
6. Warner, T.G., Dambach, L.M., Shin, J.H., and O'Brien, J.S. (1981) *J. Biol. Chem. 256,* 2952–2957.
7. Tanaka, M., Yonekura, R., Iio, T., and Tabata, T. (1984) *Lipids 19,* 714–720.
8. Brown, M.S., Kovanen, P.T., and Goldstein, J.L. (1981) *Nature 212,* 628–635.
9. Allen, K.G.D., and Klevay, L.M. (1978) *Atherosclerosis 29,* 81–93.
10. Harvey, P.W., and Allen, K.G.D. (1981) *J. Nutr. 111,* 1855–1858.
11. Lei, K.Y. (1983) *J. Nutr. 113,* 2178–2183.
12. Lefevre, M., Keen, C.L., Lonnerdal, B., Hurley, L.S., and Schneeman, B.O. (1985) *J. Nutr. 115,* 359–368.
13. Tanaka, M., Iio, T., and Tabata, T. (1987) *Lipids 23,* 1016–1019.
14. Brecher, P., Chobanian, J., Small, D.M., and Chobanian, A.V. (1976) *J. Lipid Res. 17,* 239–247.
15. Hirado, M., Iwata, D., Niinobe, M., and Fujii, S. (1981) *Biochim. Biophys. Acta 668,* 21–27.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
17. Gianetto, R., and De Duve, C. (1955) *Biochemistry 59,* 433–455.

18. Shibko, S., Aram, V., and Tappel, A. (1965) *Biochem. J. 95*, 731-741.
19. Sandell, E.B. (1965) in *Colorimetric Determination of Traces of Metals*, 3rd ed., p. 451, Interscience, New York and London.
20. Klemets, R., and Lundberg, B. (1984) *Lipids 19*, 692-698.
21. Laemmli, U.K. (1970) *Nature 227*, 680-685.
22. Deykin, D., and Goodman, D.S. (1962) *J. Biol. Chem. 237*, 3649-3656.
23. Shinomiya, M., Matsuoka, N., Shirai, K., Saito, Y., and Kumagai, A. (1979) *Atherosclerosis 33*, 343-350.
24. Khan, M.M.T., and Martell, A.E. (1967) *J. Am. Chem. Soc. 89*, 4178-4182.
25. Felsenfeld, G. (1960) *Arch. Biochem. Biophys. 87*, 247-251.
26. Dekker, A.O., and Dickinson, R.G. (1940) *J. Am. Chem. Soc. 62*, 2165-2171.
27. Shinar, E., Navok, T., and Chevion, M. (1983) *J. Biol. Chem. 258*, 14778-14783.

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Cheek Cell Fatty Acids as Indicators of Dietary Lipids in Humans

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Analysis of cheek cell lipids has been suggested as a noninvasive method for monitoring the fatty acid composition of diets in humans. In a pilot study conducted to determine the validity of the method, cheek cell samples were collected from subjects consuming a low fat (20% of calories) diet consisting of fatty acids with either a 1.0 or 0.3 P/S ratio. Neither total lipid nor polar lipid fatty acids in cheek cells consistently reflected the P/S ratio of the diets. However, there were trends, particularly in the nonpolar lipids, suggesting that cheek cell fatty acid ratios might be useful for monitoring the fatty acid composition of the diets. The diet with the higher P/S ratio (1.0 vs 0.3) consistently resulted in cheek cell lipids with lower ratios of 18:1/saturated fatty acids and greater 18:2/20:4, 18:2/18:1 and 18:2/18:0 fatty acid ratios. ***Lipids 23,*** **131–136 (1988).**

Risk for several chronic diseases, including coronary heart disease, atherosclerosis, and breast and colon cancers is thought to be associated with the amount and/or type of fat routinely consumed (1–4). However, objective validation of an association between dietary fat and these diseases has been difficult.

A major difficulty encountered in human studies has been the problem of reliably determining dietary lipid status. Most studies rely on current or retrospective reports of dietary intake. A more objective biochemical measure of fat intake would be useful for replacing or validating dietary reports in epidemiologic and intervention studies. Data presented in two recent studies suggest that cheek cells (buccal epithelial cells) might serve as a tissue lipid source that reflects dietary lipid status (5,6).

Although erythrocyte fatty acids (7,8), adipose tissue fatty acids (9–11) and serum lipids (9) have been suggested as indices of usual dietary lipid intake, there are problems with each of these. Serum cholesterol has been widely assumed to be a reflection of dietary fat intake but a direct relationship has yet to be proven (9). Serum cholesterol is influenced by changes in body weight, levels of total fat, saturated fat and, to a lesser degree, dietary cholesterol. Change in any or all of these components can be expected to affect serum cholesterol (9). What is more, there is a significant intra-individual variation in serum cholesterol levels over time (9). Adipose tissue generally is accepted as a measure of long-term fatty acid composition of diet and erythrocytes of medium- or shorter-term status. However, all require invasive techniques for sample collection and may be difficult to use in large field studies. Further, neither erythrocyte nor adipose tissue levels may be suitable for confirming reported short-term changes in lipid consumption such as might be desired in clinical trials or community intervention studies. A tissue with rapid turnover such as cheek cells was speculated to be more likely to reflect current diet than either erythrocyte or fat stores.

Compared with other biological measures of lipid intake, cheek cells offer several potential advantages. The method is relatively noninvasive, modest in cost and appears to be readily adaptable for use in large-scale field studies. In this paper, we report on the development, standardization and testing of methods used for the comparison of cheek cell lipids and dietary lipids.

METHODS

The study was performed as part of a larger project (12) conducted in collaboration with personnel from the National Cancer Institute and the Lipids Laboratory of the Human Nutrition Research Center of the U.S. Department of Agriculture, Beltsville, MD, and the Chemistry and Biochemistry Department of the University of Maryland, College Park, MD. The data presented herein represent 23 of the women in the original study (12) who agreed to provide cheek cell samples. Student's t-test was used for statistical comparison of cheek cell components. Complete details on the subjects who participated in the study are in the original paper (12).

During the study, subjects were permitted only foods prepared in the Human Nutrition Study Facility of the Beltsville Human Nutrition Research Center (BHNRC). On weekdays, the morning and evening meals were eaten in the BHNRC dining facility, and each subject was provided with an appropriate lunch. On weekends, subjects were given prepackaged meals prepared in the BHNRC facility.

All of the meals consisted of commonly available foods designed to yield a high (1.0) or low (0.3) polyunsaturated to saturated (P/S) dietary fatty acid ratio. All subjects were maintained on a high-fat diet (40% of calories) for four menstrual cycles and switched to a low-fat diet (20% of calories) for four additional menstrual cycles. Two cheek cell samples were collected from each subject, four days apart, during the mid-luteal phase of the last menstrual cycle of the low-fat dietary regimen.

Cheek cell samples were collected in the morning before eating. Subjects were asked not to brush their teeth, wear lipstick, gargle or use mouthwash prior to collection. Subjects rinsed their mouths three times with distilled water, swishing vigorously, and discarded the rinses. The study nurse then gently scraped the inside cheek just above the occlusal line three times on each side of the mouth using a plastic spoon. The cell residue on the spoon was washed off with distilled water into the collection cup. The subject rinsed a final time with distilled water and added this rinse into the collection cup. Gentamycin Sulfate (0.5 mg) was added to the sample, and it was kept on ice. Within two hr of collection, the suspensions were centrifuged at 12,000 × g × 30 min at 4 C. After discarding the supernatant, an estimate of the surface area of the pellet at

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Abbreviations: FAME, fatty acid methyl esters; GCGLC, glass capillary gas liquid chromatography; NPL, nonpolar lipids; PL, polar lipids; TLC, thin layer chromatography.

the bottom of the transparent tube was obtained, and the tube containing the pellet was capped and placed at −80 C overnight.

Homogenization and extraction. The frozen pellet was transferred with the aid of a spatula to a Potter-Elvejhem type tissue grinder, 1 ml of water was added, and the pellet was homogenized at 3,000 rpm with 10 passes of a motor-driven Teflon pestle. The homogenate was transferred to a volumetric flask using distilled water to rinse the homogenizer and to dilute the homogenate to 2 ml. After resuspending the contents, a 120 μl aliquot of the homogenate was stored at −20 C for protein and nucleic acid assays, and the remaining volume was noted and used to obtain a total lipid extract essentially as described by Folch et al. (13), except that butylated-hydroxytoluene (100 μg) was added to each sample, and the extract was filtered and concentrated without employing the traditional washing steps. All concentration steps were conducted with the exclusion of oxygen.

Two cheek cell pellets, isolated four days apart, were obtained from each subject. The lipid extract derived from the first set of pellets (Study I) was separated into polar and nonpolar lipid fractions, which were analyzed for fatty acid methyl esters (FAME) using an acidic catalyst. The total lipids derived from the second set of pellets (Study II) were analyzed for fatty acid composition after converting one portion of the lipid extract into FAME using an alkaline catalyst and converting the second portion of the lipid extract into FAME with an acidic catalyst.

Nonpolar and polar lipids. Silicic acid chromatography conducted essentially as described by Rouser et al. (14) was used to separate total lipids isolated in Study I into two fractions. The total lipid dissolved in a minimum volume of chloroform was applied to a column containing 3 g of Biosil A (Calbiochem, LaJolla, CA) and chloroform (30 ml) and methanol (30 ml) was used to elute the nonpolar and polar lipid fractions, respectively. After adding 10 μg of the internal standard (methyl heneicosanoate, 21:0), the fractions were concentrated under a stream of nitrogen and dissolved to a known volume with methylene chloride. Approximately 10% of each fraction was stored at −20 C for subsequent thin layer chromatography (TLC) studies, and the rest was converted to FAME, using an acidic catalyst, for analysis by glass capillary gas liquid chromatography (GCGLC), as described below.

Total lipids. Twenty μg of the internal standard (21:0) was added to the total lipid extract derived from the second set of cheek cell samples (Study II). After storing ca. 10% of the sample at −20 C for TLC studies, the remainder was divided into two portions. One portion was analyzed as FAME by GCGLC after transesterifying that portion with an alkaline catalyst; the second portion was analyzed after it was reacted with an acidic catalyst.

Preparation and purification of FAME. Lipids transesterified under acidic conditions were dissolved in 0.5 ml of methylene chloride and 2 ml of anhydrous methanolic HCl (1.5 N). After heating the samples for 24 hr at 80 C in tubes sealed with Teflon-lined caps, the FAME were extracted and purified as described previously (15), except that no attempt was made to separate FAME from dimethylacetals.

Lipids transesterified under alkaline conditions were dissolved in 1 ml of tetrahydrofuran and 2 ml of anhydrous methanol, containing 0.5 M $NaOCH_3$ and heated at 50 C for 10 min in tubes sealed with Teflon-lined caps. After adding 0.1 ml of glacial acetic acid, 5 ml of water and 5 ml of hexane, the FAME were extracted into the hexane layer and filtered through a small bed of anhydrous sodium sulfate containing 10% potassium bicarbonate, essentially as described by Christie (16). The filtered hexane extract was concentrated, and the crude FAME were purified by TLC as described (15).

Glass capillary gas liquid chromatography. Purified FAME were analyzed by GCGLC using three different columns installed in Hewlett-Packard (Avondale, PA) chromatographs equipped with flame ionization detectors. All samples were analyzed on a 25 m × 0.25 mm ID fused silica column that was coated with SP-2340 (Quadrex, New Haven, CT) and installed into a model 5880A GLC. The column temperature was maintained at 175 C for five min and then programmed at a rate of 0.75 C/min to a final temperature of 200 C, which was maintained for 10 min.

To facilitate the identification of FAME, all samples also were analyzed on a 12.5 m × 0.2 mm ID fused silica column, which was coated with a cross-linked dimethyl silicone (Hewlett-Packard, Avondale, PA) and installed into a model 5840 GLC. Several analyses at different constant temperatures and under temperature-programmed conditions were conducted for each sample. In addition, some samples were analyzed as previously described (15) on a 15 m × 0.25 mm ID glass capillary column coated with SP-2340. Other conditions pertinent to the operation of GCGLC column were similar to those described (15).

Identification of components was based on the retention times obtained for individual components compared to the retention times observed for available standards (NuChek, Elysian, MN; and Supelco, Bellefonte, PA). Quantitative data were derived with the aid of integrator-processors associated with the instruments, using standard mixtures of FAME to establish response factors for components of interest and using the internal standard to help convert the peak areas into μg of FAME.

Thin layer chromatography. Lipid classes were identified using TLC and densitometry. Premade Plates, containing a preabsorbent area and 10 channels (LK 5D, Linear K Silica Gel), purchased from Whatman, Inc. (Clifton, NJ) were developed in n-heptane/diethyl ether/formic acid (85:15:1, v/v/v) or in chloroform/ethanol/water/triethylamine (30:34:8:35, v/v/v/v) to resolve the nonpolar or polar lipids, respectively. Following development, components were visualized by charring essentially as described by Bitman et al. (17) and analyzed using a Kratos/Schoeffel Model SD-3000 spectrodensitometer (Westwood, NJ) equipped with a Hewlett-Packard 3390A Reporting Integrator (Avondale, PA).

Protein and nucleic acid determination. Protein was determined essentially as described by Hess and Lewin (18) using 200 μl aliquots of 50-fold dilutions of the original homogenates. The absorbance of duplicate samples was read at 750 nm in a Guilford Spectrophotometer (Oberlin, OH) and converted to protein concentration with the aid of a standard curve developed using bovine serum albumin, fraction V, purchased from Sigma Chemical Co. (St. Louis, MO).

To estimate the nucleic acid content in the cheek cell

homogenates, the absorbance of 1 ml aliquots of 50-fold dilutions of the original homogenates was measured at 260 nm and at 280 nm. An estimate of the nucleic acid content in the cuvette was obtained from the absorbance values and the following equation based on the extinction coefficients determined by Warburg and Christian (19):

$$\frac{A_{260} - [A_{280}\ (0.5728)]}{36.5942} = \text{mg nucleic acid/ml}$$

RESULTS

In preliminary experiments, we examined cheek cells pelleted at various g forces, including those recommended in the literature (5,6). Based on the yield of total FAME, as well as analysis of cheek cells by phase contrast microscopy, a considerable number of cells remained in the supernatant at forces less than 12,000 × g for 30 min. The cheek cell populations were heterogeneous, consisting of some single cells as well as several populations of contiguous cells. Based on dye exclusion tests using trypan blue (20), 10–30% of the pelleted cells did not take up the dye.

We also noted variable levels of contamination of cell pellets with microorganisms and food particles. The content and composition of FAME was affected dramatically when food particles were present but there was no obvious effect from contamination with microorganisms. In the main studies reported below, samples that obviously were contaminated with food particles, based on visual inspection, (Study I: 4 P/S 1.0 samples and 3 P/S 0.3 samples) or extremely high lipid content and unusual lipid profile (Study II: one P/S 1.0 sample), were excluded from the summary data presented.

Data for pellet size, protein content and μg of total FAME of cheek cell preparations examined in Study I and Study II are summarized in Table 1. As can be seen, there was considerable individual to individual variation and there were no statistically significant differences between diets for these parameters. The amount of cheek cells obtained using our methods was adequate for the routine analysis of nucleic acid content (data not shown) as well as protein and FAME. Based on the crude assay we used, nucleic acid was present in cheek cells at a level of about 25 μg/mg of protein.

Based on TLC, the cheek cell lipids contained relatively large amounts of triglycerides and unesterified fatty acids, variable amounts of cholesterol, relatively low levels of traditional phospholipids and a number of unidentified lipid classes. However, sample to sample variation was extremely high, suggesting that absolute levels of lipid classes were unrelated to protein level or pellet size. Nevertheless as total FAME, the nonpolar lipid fraction predominated for all samples examined and represented about 60% of the total lipid FAME.

In Study I, the cheek cell lipids were separated into nonpolar (NPL) and polar lipids (PL) before they were transesterified and analyzed by GCGLC. The major FAME in these samples, as weight percent of the total components that could be identified tentatively, are displayed in Table 2. In general, the FAME of the NPL and PL were similar but not identical. Compared to NPL, the PL contained higher percentages of 20:4 and 22:6 as well as saturated fatty acids with chain lengths greater than 18 carbon atoms. In contrast, the PL values for 14:0, 16:0 and 18:0 tended to be lower than those observed in the NPL fraction.

There was no statistically significant effect of dietary P/S ratio on the cheek cell FAME pattern. Nevertheless, there were trends toward a higher percentage of 18:2 and lower percentages of 18:1 and 20:4 in both the NPL and PL fraction derived from subjects who consumed the higher P/S diet. The extent to which factors other than sample variation may have obscured potentially significant differences was of concern. The presence of extremely long chain saturated fatty acids, particularly in the PL fraction, suggested that these samples contained amide-linked fatty acid lipid classes, which may have complicated the data by diluting out potentially significant effects of diet.

To distinguish between amide-linked and ester-linked fatty acids, in Study II the cheek cell lipids were converted to methyl esters in two ways. Aliquots were transesterified using an alkaline ($NaOCH_3$) catalyst and compared to those submitted to an acid catalyst (HCl-methanol). In addition, since our TLC studies revealed only low levels of typical phospholipids and as similar FAME patterns were observed for NPL and PL fractions, the total lipid was used as the source of FAME.

The data obtained for the major FAME in Study II are summarized in Table 3. As was expected, the percentages of long chain saturated fatty acids were considerably

TABLE 1

Pellet Size, Protein and Total Fatty Acid Methyl Esters of Cheek Cells Isolated From Subjects on 0.3 or 1.0 P/S Diets[a]

	Study 1		Study 2	
Diet P/S	1.0 (N = 7)	0.3 (N = 9)	1.0 (N = 11)	0.3 (N = 12)
Pellet (mm^2)	106.0 ± 16	113.0 ± 29	98.0 ± 23	107.0 ± 20
Protein (mg)	2.7 ± 1.4	3.6 ± 2.2	2.2 ± 1.6	2.3 ± 1.4
FAME (μg)	256.0 ± 92	254.0 ± 126	217.0 ± 65	248.0 ± 60

[a]Values are averages ± standard deviations. FAME, total fatty acid methyl esters obtained using acidic esterification conditions; N, number of individual cheek cell preparations examined.

TABLE 2

Fatty Acid Composition of Human Cheek Cell Lipids Derived from Subjects on Two Diets: Study I[a,b]

	Nonpolar lipids		Polar lipids	
Diet P/S	1.0 (N = 7)	0.3 (N = 9)	1.0 (N = 7)	0.3 (N = 9)
14:0	3.7 ± 1.2	3.4 ± 2.3	1.6 ± 0.4	1.5 ± 1.3
16:0	26.9 ± 1.1	25.3 ± 3.8	19.6 ± 2.3	18.9 ± 3.2
Σ16:1 *cis* area	6.7 ± 2.7	6.9 ± 1.9	4.9 ± 1.2	4.1 ± 0.9
18:0	19.7 ± 8.0	17.9 ± 2.8	15.4 ± 1.5	15.9 ± 2.0
18:1 *trans* area	1.6 ± 0.8	2.9 ± 2.6	1.1 ± 1.0	0.8 ± 0.4
Σ18:1 *cis* area	18.8 ± 5.4	21.1 ± 3.6	20.9 ± 3.7	23.2 ± 2.6
18:2 *trans* area	0.4 ± 0.2	0.5 ± 0.3	0.3 ± 0.1	0.3 ± 0.1
18:2(n-6)	13.8 ± 5.0	11.0 ± 2.6	12.1 ± 3.2	11.9 ± 2.1
20:4(n-6)	0.8 ± 0.2	1.0 ± 0.4	2.4 ± 0.7	2.7 ± 0.6
22:6(n-3)	t	t	0.5 ± 0.0	0.6 ± 0.4
Σn-6 poly	15.0 ± 5.6	12.6 ± 3.8	16.1 ± 2.9	15.2 ± 2.6
Σn-3 poly	0.6 ± 0.3	0.5 ± 0.1	0.5 ± 0.2	1.2 ± 1.1
Σsat>18 carbon	1.7 ± 1.1	3.0 ± 1.9	8.3 ± 1.6	7.4 ± 2.2
Σsat 14,16,18	50.3 ± 14	46.5 ± 6.7	36.0 ± 3.6	36.3 ± 4.9
Σpoly	15.4 ± 5.8	13.1 ± 4.2	16.4 ± 3.2	15.4 ± 3.1

[a]Values are averages and standard deviations for FAME as wt % of the total normalized FAME. N, number of individual cheek cell preparations examined; t, less than 0.1%.

[b]Other tentatively identified components, not included in the table, were dimethylacetals, odd-chain and branched-chain fatty acids. In addition, there were some components for which a tentative identification could not be assigned.

TABLE 3

Fatty Acid Composition of Human Cheek Cell Lipids Derived from Subjects on Two Diets: Study II[a,b]

	HCl-Methanol		$NaOCH_3$	
Diet P/S	1.0 (N = 10)	0.3 (N = 12)	1.0 (N = 10)	0.3 (N = 12)
14:0	2.3 ± 0.9	2.2 ± 1.0	2.3 ± 1.0	2.5 ± 0.6
16:0	22.3 ± 2.1	22.2 ± 3.3	15.0 ± 2.6	15.9 ± 5.3
Σ16:1 *cis* area	6.9 ± 1.3	6.7 ± 1.0	13.6 ± 3.7	14.5 ± 5.2
18:0	15.7 ± 1.6	14.3 ± 2.0	9.8 ± 1.9	9.1 ± 2.8
18:1 *trans* area	0.9 ± 0.5	0.5 ± 0.5	0.4 ± 0.5	0.2 ± 0.2
Σ18:1 *cis* area	23.1 ± 2.6	26.0 ± 4.2	24.9 ± 3.5	25.3 ± 4.4
18:2 *trans* area	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2
18:2(n-6)	12.5 ± 1.1	11.5 ± 2.2	15.1 ± 3.6	12.6 ± 2.6
20:4(n-6)	1.9 ± 0.4	2.3 ± 0.9	2.0 ± 0.8	2.2 ± 0.9
22:6(n-3)	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
Σn-6 poly	14.8 ± 1.1	14.4 ± 2.8	17.4 ± 4.6	15.4 ± 2.3
Σn-3 poly	0.9 ± 0.5	0.9 ± 0.3	0.2 ± 0.3	0.2 ± 0.4
Σsat>18 carbon	5.0 ± 0.8	4.5 ± 1.2	1.0 ± 0.8	1.1 ± 0.7
Σsat 14,16,18	40.2 ± 3.2	38.9 ± 3.7	28.0 ± 2.4	27.4 ± 6.1
Σpoly	15.3 ± 1.6	15.2 ± 3.0	17.9 ± 5.0	15.6 ± 2.3

[a]Values are averages and standard deviations for FAME as wt % of the total normalized FAME. N, number of individual cheek cell preparations examined.

[b]Other tentatively identified components, not included in the table were dimethylacetals, odd-chain and branched-chain fatty acids. In addition, there were some components for which a tentative identification could not be assigned.

lower in samples transesterified with $NaOCH_3$, compared to those submitted to the HCl-methanol method. In general, the two methods yielded the same percentages of mono- and polyunsaturated FAME, with the exception of the larger percentage of the component tentatively identified as *cis*-16:1 in the samples transesterified with $NaOCH_3$.

Although the results of Study II were encouraging, there were no statistically significant differences in the observed patterns that could be ascribed to differences

TABLE 4

Ratios of Selected FAME in Human Cheek Cell Lipids[a]

	Study I						Study II			
	Nonpolar lipid		Polar lipid		Total lipid		HCl-Methanol		$NaOCH_3$	
Diet P/S	1.0 (N = 7)	0.3 (N = 9)	1.0 (N = 7)	0.3 (N = 9)	1.0 (N = 7)	0.3 (N = 9)	1.0 (N = 10)	0.3 (N = 12)	1.0 (N = 10)	0.3 (N = 12)
18:2/20:4	21.3 (3.4)	12.5 (5.2)	5.5 (2.0)	4.4 (1.0)	10.7 (4.0)	8.2 (2.4)	6.8 (2.0)	6.0 (3.4)	9.6 (4.5)	8.8 (7.6)
18:2/18:1	0.73 (0.11)	0.53 (0.11)	0.58 (0.10)	0.52 (0.12)	0.65 (0.09)	0.52 (0.10)	0.54 (0.07)	0.45 (0.10)	0.60 (0.07)	0.51 (0.10)
18:2/18:0	0.86 (0.48)	0.62 (0.17)	0.80 (0.24)	0.76 (0.15)	0.81 (0.38)	0.66 (0.16)	0.82 (0.14)	0.75 (0.20)	1.6 (0.4)	1.5 (0.7)
18:1/Sat[b]	0.42 (0.18)	0.46 (0.13)	0.59 (0.14)	0.64 (0.10)	0.47 (0.16)	0.56 (0.14)	0.58 (0.09)	0.68 (0.16)	0.90 (0.21)	0.98 (0.28)
PUFA/Sat[c]	0.35 (0.21)	0.30 (0.13)	0.46 (0.14)	0.45 (0.13)	0.39 (0.17)	0.36 (0.12)	0.39 (0.06)	0.40 (0.10)	0.65 (0.24)	0.60 (0.24)

[a]Values are averages of ratios in individual samples with the standard deviation shown in parentheses. Values for the total lipid were derived from summing the μg of FAME in nonpolar and polar lipids.

[b]Sat, sum of 14:0 + 16:0 + 18:0.

[c]PUFA, sum of n-3 and n-6 polyunsaturated fatty acids.

in dietary P/S ratio. The trends noted in Study I also were evident in Study II; that is, cheek cell lipids derived from subjects on the higher P/S diet contained slightly higher percentages of 18:2 and slightly lower percentages of 18:1 and 20:4.

To compare the data obtained in both studies, we calculated ratios for FAME. The values presented in Table 4 are for those ratios that consistently displayed a trend that appeared to be attributable to diet. Regardless of the method of analysis, the average values obtained for 18:2/20:4, 18:2/18:1 and 18:2/18:0 were always greater for cheek cell lipids derived from subjects consuming the higher P/S diet. There was also a trend for the polyunsaturated fatty acid/14:0 + 16:0 + 18:0 (Sat) to be higher in cheek cell lipids of subjects on the higher P/S diet. However, the 18:1/(Sat) tended to be lower in the women on the higher P/S diet.

DISCUSSION

Despite our efforts to standardize procedures for sampling cheek cells, there were large sample-to-sample variations in all parameters analyzed. Nevertheless, a number of trends were observed that suggest diet may influence observed FAME patterns in cheek cell lipids. Regardless of the procedures used to analyze the lipids, FAME of cheek cell preparations derived from subjects on the higher P/S diet displayed lower percentages of 18:1 and 20:4, a slightly higher percentage of 18:2, higher ratios for 18:2/20:4, 18:2/18:1 and 18:2/18:0 and a lower ratio for 18:1/(Sat).

The 18:2/18:1 values observed in cheek cell lipids (Table 4) were similar to the ratios calculated for the diets consumed by subjects in these studies (0.76 and 0.46 for the high and low P/S diets, respectively). These results suggest that some aspects of diet fatty acid composition may have a direct effect on cheek cell lipid composition. In contrast, the trends observed for 18:2/20:4 and 18:1/(Sat) in our studies are more difficult to interpret.

The ratio of 18:1/(Sat) was calculated to be 1.49 and 0.71 for the high and low P/S diets, respectively. Thus, the diet with the greater percentage of 18:2 and the greater ratio of 18:1/(Sat) apparently resulted in cheek cell lipids with lower values of 18:1 and 20:4, lower ratios of 18:1/(Sat) but higher ratios of 18:2/20:4.

The inverse relationship between the dietary percentage of 18:2 and the observed values for 18:1 and 20:4 in cheek cell lipids may be a reflection of the differential effect of the two diets on desaturase activities. A similar relationship between dietary 18:2 and 18:1 values in human adipose tissue triglycerides has been reported (10,21,22). While the difference in 20:4 in the cheek cells may reflect dietary levels, it is possible that these values also reflect an effect of the diet on desaturase activities. It is well known that the level and type of dietary fat may influence key enzymes involved in fatty acid biosynthesis and desaturation (23). For example, Herodek and Csakvary (24) have reported that saturated fatty acids increase desaturase activity in liver and adipose tissue of mice. In contrast, Jeffcoat and James (25) have shown that linoleic acid is a potent inhibitor of stearoyl-CoA desaturase activity in rat liver. In addition, Hagve and Christophersen (26) have reported higher Δ-6 desaturase activity in isolated liver cells derived from rats fed hydrogenated coconut oil compared with liver cells derived from rats fed soybean oil or standard rodent pellets. Moreover, polyunsaturated fatty acids have been shown to have a selective dampening effect on other lipogenic enzymes, including acetyl-CoA carboxylase (27). We believe that the data observed in our studies may be a reflection of similar controls on lipogenic enzymes in humans.

In contrast to the reports by McMurchie et al. (5,6), we found virtually identical P/S ratios in polar lipids of cheek cells. It is possible that differences in dietary P/S ratio during periods of low-fat intake (as was the case in our study) may have no visible effect on polar lipid FAME patterns. Moreover, since polar lipids are largely structural components, which also may participate in important cellular functions, it is likely that relatively rigorous metabolic controls act to minimize any drastic alteration in the composition of these constituents. In this regard, it should be noted that others have failed to observe differences in adipose tissue phospholipid P/S ratios of subjects on different P/S diets (10).

Whereas polar lipids are resistant to dietary manipulation, this is not the case for triglycerides. Alterations in tissue triglyceride fatty acid composition resulting from dietary lipid differences have been noted in several studies, including those in humans (10). In our studies,

it seems relevant that the greatest differences for P/S ratios in cheek cell lipids between the low and high P/S diets were observed for nonpolar lipids in Study I and for FAME resulting from $NaOCH_3$ transesterification in Study II (Table 4). In both cases, triglycerides would have contributed substantial proportions of fatty acids to the resulting FAME analyses. Future studies directed specifically at the fatty acyl composition of cheek cell triglycerides may shed light on this point.

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REFERENCES

1. Keys, A. (1980) *Seven Countries. A Multivariate Analysis of Death and Coronary Heart Disease*, Harvard University Press, Cambridge, MA.
2. Grundy, S.M., Bilheimer, D., Blackburn, H., Brown, W.V., Kwiterovich, P.O. Jr., Mattson, F., Schonfeld, G., and Weidman, W.H. (1982) *Circulation 65*, 938A.
3. Kolonel, L.N., Hankin, J.H., Lee, J., Chu, S.Y., Nomura, A.M.Y., and Hinds, M.W. (1981) *Br. J. Cancer 44*, 332-339.
4. Carroll, K.K., and Khor, H.T. (1975) *Progr. Biochem. Pharmacol. 10*, 308-353.
5. McMurchie, E.J., Potter, J.D., Rohan, T.E., and Hetzel, B.S. (1984) *Nutr. Rep. Int. 29*, 519-526.
6. McMurchie, E.J., Margetts, B.M., Beilin, L.J., Croft, K.D., Vandongen, R., and Armstrong, B.K. (1984) *Am. J. Clin. Nutr. 39*, 975-980.
7. Angelico, F., Arca, M., Calviari, A., Cantafora, A., Guccione, P., Monini, P., Montali, A., and Ricci, G. (1983) *Prev. Med. 12*, 124-127.
8. Sirtori, C.R., Tremoli, E., Gatti, E., Montanari, G., Sirtori, M., Colli, S., Gianfranceschi, G., Maderna, P., Dentone, C.Z., Testolin, G., and Galli, C. (1986) *Am. J. Clin. Nutr. 44*, 635-642.
9. Berry, E.M., Hirsch, J., Most, J., McNamara, D.J., and Thornton, J. (1986) *Am. J. Clin. Nutr. 44*, 220-231.
10. Field, C.J., and Clandinin, M.T. (1984) *Nutr. Res. 4*, 743-755.
11. Beynen, A.C., Hermus, R.J., and Hautvast, J.G.A.J. (1980) *Am. J. Clin. Nutr. 33*, 81-85.
12. Jones, D.Y., Judd, J.T., Taylor, P.R., Campbell, W.S., and Nair, P.P. (1987) *Am. J. Clin. Nutr. 45*, 1451-1456.
13. Folch, J., Lees, M., and Sloan-Stanley, G. (1957) *J. Biol. Chem. 226*, 497-509.
14. Rouser, G., Kritchevsky, G., and Yamamoto, A. (1967) in *Lipid Chromatographic Analysis* (Marinetti, G.V., ed.), Vol. 1, pp. 99-162, Marcel Dekker, Inc., New York, NY.
15. Sampugna, J., Pallansch, L.S., Enig, M.G., and Keeney, M. (1982) *J. Chromatography 249*, 245-255.
16. Christie, W.W. (1982) in *Lipid Analysis*, 2nd ed., pp. 53-54, Pergamon Press, Oxford, England.
17. Bitman, J., Wood, D.L., and Ruth, J.M. (1982) *Liq. Chromatogr. 4*, 1007-1021.
18. Hess, H., and Lewin, E. (1965) *J. Neurochem. 12*, 205-211.
19. Warburg, O., and Christian, W. (1941) *Biochem. Z. 310*, 384-421.
20. Patterson, M.K. Jr. (1979) in *Methods of Enzymology* (Jakoby, W.B., and Pastan, I.H., eds.) Vol. LVIII, pp. 150-152, Academic Press, New York, NY.
21. Shepherd, J., Stewards, J.M., Clark, J.G., and Carr, K. (1980) *Br. J. Nutr. 44*, 265-271.
22. Christakis, G.J., Rinzler, S.H., Archer, M., Hashim, S.A., and Van Itallie, T.B. (1965) *Am. J. Clin. Nutr. 16*, 243-251.
23. Jeffcoat, R., and James A.T. (1984) in *Fatty Acid Metabolism and Its Regulation* (Numa, S., ed.) pp. 85-112, Elsevier, Amsterdam.
24. Herodek, S., and Csakvary, G. (1972) *Acta Biochim. Biophys. Acad. Sci. Hung. 7*, 207-213.
25. Jeffcoat, R., and James, A.T. (1978) *FEBS Lett. 85*, 114-118.
26. Hagve, T.A., and Christophersen, G.O. (1984) *Biochim. Biophys. Acta 796*, 205-217.
27. Muto, Y., and Gibson, D.M. (1970) *Biochem. Biophys. Res. Commun. 38*, 9-15.

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COMMUNICATIONS

New Oxidation Products of α-Tocopherol

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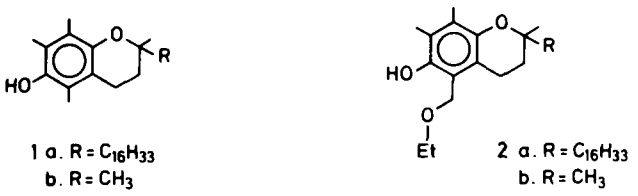
A major product of the reaction between α-tocopherol and *t*-butyl hydroperoxide in chloroform is 5-ethoxymethyl-7,8-dimethyl tocol, the source of the ethoxy group being the ethanol that is used to stabilize the chloroform. Two new products of this oxidation have now been identified as 2-(3′-hydroxy-3′,7′,11′,15′-tetramethylhexadecyl), 3-ethoxymethyl-5,6-dimethylbenzo-1,4-quinone and 5-ethoxycarbonyl-7,8-dimethyl tocol. These two compounds and another major product, 5-formyl-7,8-dimethyl tocol appear to be formed by further oxidation of 5-ethoxymethyl-7,8-dimethyl tocol.
Lipids 23, 137–139 (1988).

When α-tocopherol (**1a**) and its model compound, 2,2,5,7,8-pentamethyl-6-hydroxy chroman (**1b**) (Scheme 1), are oxidized by *t*-butyl hydroperoxide in purified chloroform to which ethanol has been added, 5-ethoxymethyl-7,8-dimethyl tocol (**2a**) and 5-ethoxymethyl-2,2,7,8-tetramethyl-6-hydroxy chroman (**2b**), respectively, are formed in amounts that vary with the concentration of ethanol (1). Oxidation does not occur in the absence of ethanol. If the reaction is performed in distilled, reagent-grade chloroform (containing 2% ethanol as stabilizer but with no additional ethanol), higher levels of **2a** and **2b** are produced together with a new dimeric quinone (Suarna, C., Craig, D.C., Cross, K.J., and Southwell-Keely, P.T., unpublished data) and other as yet uncharacterized compounds. The present work reports on the identification of several of these compounds.



SCHEME 1

MATERIALS AND METHODS

Infrared (IR) spectra were determined on a Perkin Elmer 580B spectrometer, ultraviolet (UV) spectra on a Perkin Elmer 124 double beam spectrophotometer and ^1H nuclear magnetic resonance (NMR) spectra on a Bruker CXP 300 spectrometer and a Bruker AM 500 spectrometer. NMR spectra were taken in $CDCl_3$ and are reported in parts per million downfield from tetramethylsilane as internal standard. Electron impact mass spectra were determined at 70 ev on an A.E.I. MS12 spectrometer and chemical ionization mass spectra on a Finnigan 3200 quadrupole spectrometer. High resolution molecular weights were determined on a Bruker Spectrospin Fourier Transform Ion Cyclotron Resonance spectrometer.

(±)-α-Tocopherol (**1a**, Roche Products, Sydney) was used without further treatment.

Distilled, reagent-grade chloroform (Ajax Chemicals, Sydney) was used as a solvent for the oxidation reactions.

Chloroform for thin layer chromatographic (TLC) plate elution was purified by washing with 18 M sulfuric acid and distilled water until the washings were neutral, drying (Na_2SO_4) and distilling immediately before use.

t-Butyl hydroperoxide (70%-EGA Chemie, Steinheim, FRG) was purified by the sodium salt method (2). Purity (iodometrically) was 95%.

The spiro dimer and spiro trimer of ***1a***, 5-formyl-7,8-dimethyl tocol (**7**); ***2a***, α-tocopheryl quinone; and 8a-ethoxy-α-tocopherone were prepared as reference compounds by known methods. (1,3–6).

*Oxidation of **1a** by t-butyl hydroperoxide.* A typical reaction was as follows: To **1a** (1011 mg, 2.25 mmol) in reagent grade chloroform (150 ml) was added *t*-butyl hydroperoxide (223 mg, 2.48 mmol) and the solution refluxed for 3 hr. The solution was then washed with 5% ferrous sulfate solution (6 × 30 ml) and distilled water (7 × 30 ml) and dried (Na_2SO_4), and the solvent was removed in vacuo. The residue was chromatographed on thin layers of Silica Gel GF_{254} (solvent: light petroleum (bp 60–80 C) ethyl acetate [9:1, v/v]), the products were located under UV light and eluted with purified chloroform and the solvent was removed under a stream of nitrogen and weighed. The following products were identified by comparison of their UV and ^1H NMR spectra with those of the prepared reference compounds; **7**, R_f 0.61; **2a**, R_f 0.53 (582 mg, 58%); **1a**, R_f 0.42. Products of unknown composition were isolated with R_f 0.87, 0.75, 0.69, 0.24, 0.17, 0.12 and 0.0.

The overlapping bands with R_f 0.12 (pink) and R_f 0.0 (yellow) were combined, rechromatographed on Silica Gel GF_{254} (solvent, light petroleum [bp 60–80 C] chloroform [2:8, v/v]) and resolved into three bands with R_f 0.21 (pale yellow, 11 mg, 1.1%), 0.34 (pink-orange, 24 mg, 2.4%) and 0.56. The compound with R_f 0.56 has been identified as a dimeric quinone (Suarna, C., Craig, D.C., Cross, K.J., and Southwell-Keely, P.T., unpublished data), while the compound with R_f 0.34 is under investigation.

2-(3′-Hydroxy-3′,7′,11′,15′-tetramethylhexadecyl)-3-ethoxymethyl-5,6-dimethyl-benzo-1,4-quinone (**4**) (R_f 0.21), a pale yellow oil, was identified by IR (KBr) 3450 (OH), 2930 (CH_3), 1650 (C=C–C=O), 1465, 1380,

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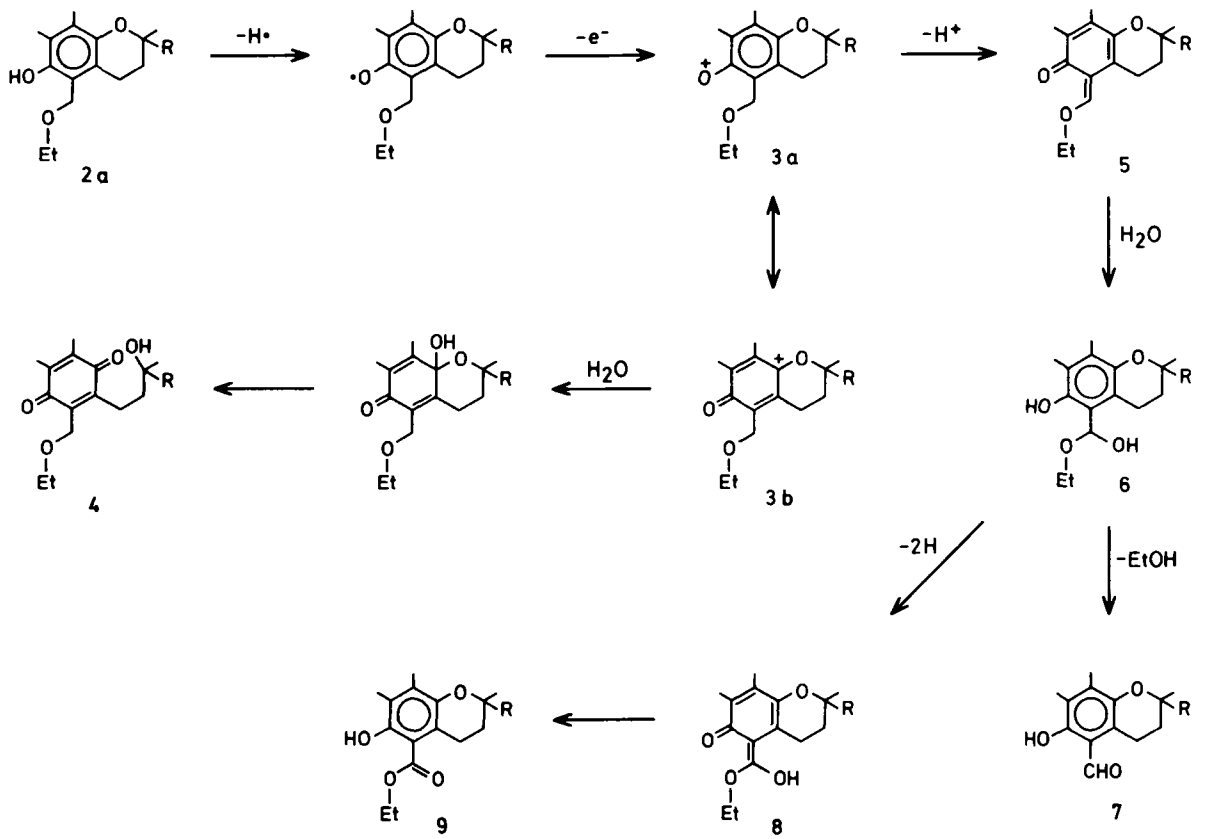
1305, 1100 (C-O-C) cm^{-1}; UV (hexane) λ_{max} 258, 267 (sh) nm; ^{1}H NMR ($CDCl_3$) δ 0.85 (m, 12H), 1.21 (t, 3H, J = 7.0 Hz, *CH_3*-CH_2-0-), 1.25 (broad m, 24H), 1.60 (m, 2H, =C-CH_2-*CH_2*-), 2.018 *(s, 3H,* =C-*CH_3*), *(s, 3H,* =C-*CH_3*), 2.65 (m, 2H, =C-*CH_2*-), 3.65 (q, 2H, J = 7.0 Hz, CH_3-*CH_2*-0-), 4.41 (s, 2H, =C-*CH_2*-0-) ppm. Irradiation of the singlet at 4.41 ppm led to an NOE on the multiplet at 2.65 ppm and on the quartet at 3.65 ppm, thus proving that the ethoxymethyl and methylene groups were attached to adjacent carbon atoms of the quinone ring. MS (ei) m/z (rel int) 490 (M^+, 0.5), 489 (0.5), 488 (1), 472 (0.5) (M^+-H_20), 444 (2) (M^+-C_2H_5OH), 442 (1), 431 (5), (M^+-C_2H_5-0-CH_2), 428 (3), 426 (1), 368 (3), 321 (50), 279 (52), 250 (13), 236 (21), 194 (100); MS (ci, methane) m/z (rel int) 491 (MH^+, 1), 490 (1), 489 (4), 473 (100) (MH^+-H_2O), 471 (14), 457 (4), 445 (10) (MH^+-C_2H_5OH), 427 (9) (MH^+-C_2H_5OH-H_2O). The normal methane adduct ions were noted at 519 $(M+29)^+$ and 531 $(M+41)^+$, confirming that the molecular weight was 490. Exact mass calculation for $C_{31}H_{54}O_4$ 490.4328; found, 490.4354.

*Isolation of 5-ethoxycarbonyl-7,8-dimethyl tocol (**9**).* Compound **2a** (300 mg), which had been kept in the refrigerator for two months, was rechromatographed on thin layers of Silica Gel GF_{254} [solvent, light petroleum (bp 60–80 C) ethyl acetate (9:1, v/v)]. Three bands were obtained with R_f 0.49 (**2a**), 0.60 (**7**) and 0.63.

Compound **9**. R_f 0.63, a bright yellow oil, was purified further by chromatography on Silica Gel GF_{254} [solvent, light petroleum bp 60–80 C/CCl_4 (16:9) ethyl acetate (99:1)] and was identified by IR (KBr) 3400 (weak H-bonded OH), 2930 (CH_3), 1656 (C=0), 1470, 1375, 1320, 1205, 1095 (C-O-C), 1030, 800 cm^{-1}; UVλ_{max} 260, 268, 350 nm; ^{1}H NMR ($CDCl_3$) δ 0.86 (m, 12H), 1.25 (broad m, 24H), 1.42 (t, 3H, J = 7.1 Hz; *CH_3*-CH_2-0), 1.72 (m, 2H, Ar-CH_2-*CH_2*-), 2.16 (s, 3H, Ar-*CH_3*), 2.18 (s, 3H, Ar-*CH_3*), 3.00 (t, 2H J = 6.9 Hz, Ar-*CH_2*-), 4.41 (q, 2H, J = 7.1 Hz, CH_3-*CH_2*-0-), 11.3 (s, 1H, OH); MS (ei) m/z (rel int) 488 (M^+, 100), 442 (47) (M^+-C_2H_5OH). Exact mass calculation for $C_{31}H_{52}O_4$ 488.4111; found, 488.4069.

*Oxidation of **2a** by t-butyl hydroperoxide.* To **2a** (36 mg) in reagent-grade chloroform (5 ml) was added *t*-butyl hydroperoxide (9 mg), and the solution was refluxed at 60 C for 3 hr. The solution was cooled, washed with 5% ferrous sulfate (2 × 10 ml), water (3 × 10 ml) and dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo. The residue was chromatographed on thin layers of silica gel [solvent, light petroleum (bp 60–80 C)/ethyl acetate (9:1)], the products were located under UV light and eluted with chloroform, and the solvent was removed under a stream of nitrogen and weighed. The majority of products of this reaction have yet to be identified but their R_{fs} were as follows: R_f 0.65 unknown (0.6 mg; 2%); R_f 0.62 unknown (5 mg; 15%); R_f 0.61 unknown (2 mg; 5%); R_f 0.59, **7** (10 mg; 28.5%); R_f 0.54 **2a** (4 mg; 12%) R_f 0.51 unknown (6 mg; 16%); R_f 0.45 unknown (3 mg; 8.5%); R_f 0.15 unknown (4 mg; 12%).



SCHEME 2

Compound **7** was identified by UV λ_{max} 238 (ϵ 7012), 282 (ϵ 12053), 288 (ϵ 12196), 387 (ϵ 4022) nm; IR (KBr) 3400 (very weak H-bonded OH), 2920 (CH_3), 1635 (C=0), 1460, 1380, 1310, 1095 (C-O-C) cm^{-1}; ^{1}HNMR ($CDCl_3$) δ 0.85 (m, 12H), 1.05-1.65 (broad m, 24H), 1.82 (m, 2H, 3-CH_2), 2.15 (s, 3H, Ar-CH_3), 2.17 (s, 3H, Ar-CH_3), 3.03 (t, 2H, 4-CH_2, J = 6.6Hz), 10.2 (s, 1H, CHO), 12.1 (s, 1H, OH) ppm, Irradiation of the singlet at 10.2 ppm produced an NOE on the triplet at 3.03 ppm (4-CH_2), thus proving that the aldehyde group was on position 5 of the chroman; MS (ei) m/z (rel int) 444 (M^+ 85), 426, (21), 271 (6), 243 (12), 229 (12), 220 (47), 219 (24), 215 (18), 202 (44), 201 (100).

RESULTS AND DISCUSSION

Both compounds **4** and **9** appear to be formed by oxidation of **2a**. However, when **2a** is oxidized by *t*-butyl hydroperoxide in reagent-grade chloroform, the major product by far is **7**, so that the formation of **4** and **9** represents minor pathways.

The formation of these products may be explained by assuming that **2a** is oxidized in the same way as **1a**, through a tocopheroxyl radical (7,8) to a phenoxylium species (**3a,3b**) (9,10) and a quinone methide (**5**) (11,12) (Scheme 2). Water formed during the oxidation may add to the phenoxylium ion producing the benzoquinone (**4**) or it may add to the quinone methide producing a hemi-acetal (**6**). Compound **6** may lose ethanol to form the aldehyde (**7**) or it may be oxidized to a further quinone methide (**8**), which is the tautomer of the ester (**9**). Clearly, the conditions which favor the formation of **9** are very mild.

ACKNOWLEDGMENT

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REFERENCES

1. Sumarno, M., Atkinson, E., Suarna, C., Saunders, J.K., Cole, E.R., and Southwell-Keely, P.T. (1987) *Biochim. Biophys. Acta*, in press.
2. Barnard, D., and Hargrave, K.R. (1951) *Anal. Chim. Acta 5*, 476-488.
3. Skinner, W.A., and Alaupovic, P. (1963) *J. Org. Chem. 28*, 2854-2857.
4. John, W., Dietzel, E., and Emte, W. (1939) *Z. Physiol. Chem. 257*, 173-189.
5. Fujimaki, M., Kanamaru, K., Kurata, T., and Igarashi, O. (1970) *Aqric. Biol. Chem. 34*, 1781-1787.
6. Boyer, P.D. (1951) *J. Am. Chem. Soc. 73*, 733-740.
7. Matsuo, M., Matsumoto, S., and Ozawa, T. (1983) *Org. Mag. Res. 21*, 261-264.
8. Doba, T., Burton, G.W., and Ingold, K.U. (1983) *J. Am. Chem. Soc. 105*, 6505-6506.
9. Martius, C., and Eilingsfeld, H. (1957) *Annalen 607*, 159-168.
10. Durckheimer, W., and Cohen, L.A. (1964) *J. Am. Chem. Soc. 86*, 4388-4393.
11. Skinner, W.A., and Parkhurst, R.M. (1966) *J. Org. Chem. 31*, 1248-1251.
12. Nilsson, J.L.G., Branstad, J.O., and Sievertsson, H. (1968) *Acta Pharm, Suecica 5*, 509-516.

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Detection of *cis*-Vaccenic Acid in Palm Oil by ^{13}C NMR Spectroscopy

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The NMR signals of the carbonyl and olefinic carbons of the oils of some species of palm show some relatively weak peaks at characteristic positions that have not been identified previously. These peaks are most intense in the oil of the species *Elaeis oleifera*. On the basis of the chemical shift data of the carbonyl and olefinic carbons of several synthetic monoenic triacylglycerols and of the packed-column gas chromatogram of the methyl esters of the oil of *E. oleifera*, the peaks in question are assigned to *cis*-vaccenic acid (18:1,[*cis*]-11). ^{13}C NMR spectroscopy is an effective technique for the detection of *cis*-vaccenic acid and other monounsaturated fatty acids in vegetable oils or fats.

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Cis-vaccenic acid (*cis*-11-octadecenoic acid) (1) is well known to be a component fatty acid of microorganisms and animal tissues. Although it has been identified previously in plant sources (2), its presence in vegetable oils and plant lipids has not been well investigated, probably because of its low concentration and of some difficulties in conventional analytical procedures. Recent progress in capillary gas chromatography (GC) has made it possible to detect *cis*-vaccenic acid in the presence of other isomeric monoenoic acids in plant lipids (3). A new analytical procedure for determination of *cis*-vaccenic acid in plant lipids is by gas chromatography-mass spectrometry (GC-MS) through its pyrrolidine- and bis(methylthio)-derivatives (4). By the use of picolinyl ester derivatives, the GC-MS technique has been found to be advantageous in determining both the numbers and positions of the double bonds in fatty acids (5).

In this paper, we show that isomeric monoenoic acids in triacylglycerols can be differentiated easily in the ^{13}C NMR spectra of the carbonyl and olefinic carbons and, by this technique, *cis*-vaccenic acid in the oil of certain species of palm is detected.

MATERIALS AND METHODS

The samples of palm oil used in this study were obtained from the Guthrie Research Chemara (GRC) experimental station where various species of palm are grown and from local palm oil mills. The synthetic homogeneous triacylglycerols were obtained from Sigma Chemical Co. (St. Louis, MO).

For ^{13}C NMR analysis, the palm oil was dissolved in chloroform-*d* in the volume ratio 1:4 and then filtered. The synthetic triacylglycerols or mixtures of them also were dissolved in chloroform-*d* to form 2-3 mol % solutions. The ^{13}C NMR spectra were recorded on a JEOL FX100 high-resolution Fourier transform NMR spectrometer with the probe temperature at 28 C.

Packed-column gas chromatography (GC) was carried out with a Perkin Elmer model 8310 gas chromatograph equipped with a flame ionization detector (FID). Operating conditions were 2m × 3 mm i.d. glass column packed with 10% SP-2300 on 100-120 mesh Chromosorb W; column temperature, isothermal at 200 C. The fatty acid methyl esters (FAME) were prepared by normal procedures and then injected into the GC instrument, using the on-column injection technique via a glass liner.

RESULTS AND DISCUSSION

Recently, it has been shown that the carbonyl carbons of various fatty acids at the 1,3- and the 2-positions of the triacylglycerols of palm oil (or any vegetable oil or fat) have different chemical shifts and can be distinguished in the carbon-13 NMR spectrum (6). Similarly, the olefinic carbons of acyl groups in the two glycerol positions can be distinguished (7). The carbon-13 NMR spectra of the oil of several species of palm grown at the GRC experimental station have been examined. The spectra of the carbonyl and olefinic carbons of the oils of two species (*Elaeis oleifera* and a hybrid Dura (GRC code 395) show some relatively weak peaks at characteristic positions that previously have not been identified. These peaks are most intense in the case of the oil of *E. oleifera* and appear between those of the carbonyl carbons of the oleic and the saturated acyl groups and between the two groups of peaks for the olefinic carbons of the oleic acyl group, as shown in Figures 1 and 2. These spectra suggest the new peaks belong to a monoenic fatty acid. In the sample of oil of *E. oleifera* shown in Figure 1, it is present at about 5 mol % of the total fatty acid content by estimating from the intensities of the peaks (6). The existence of this fatty acid is also detectable (<1 mol %) by carbon-13 NMR in some palm oil samples obtained from palm oil mills, where the oil would be a mixture of oils from various plantation sources or palm species.

To identify the monoenic acyl group described above, the carbon NMR spectra of several synthetic homogeneous triacylglycerols in mixtures with triolein and/or tripalmitin were obtained. The chemical shift data for the carbonyl and olefinic carbons are shown in Tables 1 and 2. The spectra of the carbonyl and olefinic carbons of a mixture of tripalmitin, triolein and trieicosenoin are shown in Figure 3. Comparison of Figures 1 and 2 with Figure 3 immediately suggests that the new peaks detected in the former may be assigned to a *cis*-11-monoene.

The gas chromatogram (packed column) of the methyl esters of fatty acids in the oil of *E. oleifera* provides the following composition (in wt %): palmitic (16:0) 15.1, palmitoleic (16:1) 1.7, stearic (18:0) 0.9, oleic (18:1) 66.2, linoleic (18:2) 15.3, linolenic (18:3) 0.6, and arachidic (20:0) 0.1. No monoenoic fatty acid longer in chain than 18:1 is detected. The peak for methyl oleate is twice as broad

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Abbreviations: FAME, fatty acid methyl esters; FID, flame ionization detector; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; GRC, Guthrie Research Chemara.

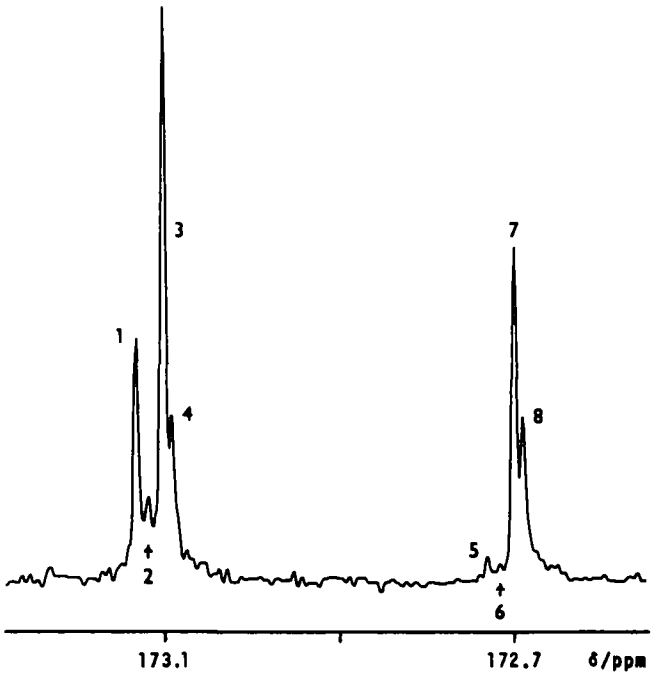

FIG. 1. 25.05 MHz ^{13}C NMR spectrum of the carbonyl carbons of the triacylglycerols in the oil of *E. oleifera* in chloroform-*d* solution, conc. 1:4 v/v, at 28 C. The group of peaks 1–4 pertains to acyl groups at the 1,3-positions, while the group of peaks 5–8 pertains to those at the 2-position. Peaks 1 and 5 belong to saturated acyl groups, peaks 3 and 7 belong to oleic (18:1,[*cis*]-9), and peaks 4 and 8 belong to linoleic (18:2,[*cis,cis*]-9-12) and linolenic (18:3,[*cis,cis,cis*]-9,12,15) acyl groups (6). Peaks 2 and 6 at δ 173.13 and 172.72 ppm, respectively, have not been identified previously.

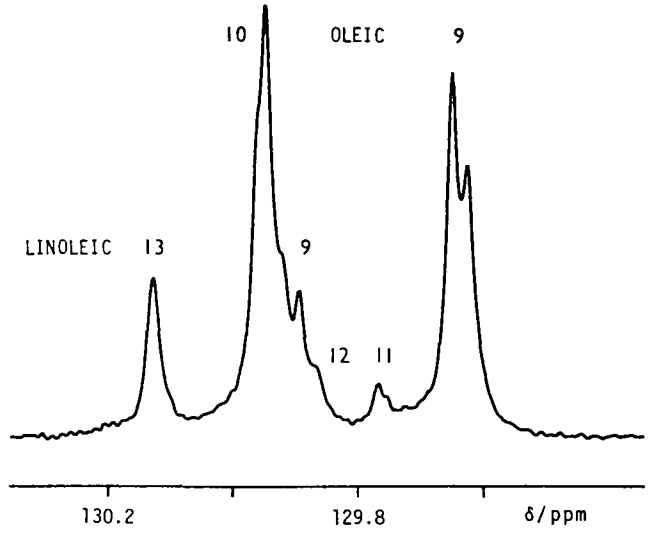

FIG. 2. 25.05 MHz ^{13}C NMR spectrum of the olefinic carbons of the triacylglycerols in the sample of the oil of *E. oleifera* described in Figure 1. Each olefinic carbon gives rise to two peaks, if resolved, because of the two glycerol positions (7). The labeling of the peaks corresponds to the positions of the olefinic carbons. The taller peak of C-9 of oleic pertains to the acyl groups at the 1,3-positions, while the lower peak pertains to that at the 2-position. The peaks of C-10 of oleic are not well-resolved; a shoulder appears on the high frequency side of the taller peak. Only the peaks of C-9 and C-13 of linoleic are shown; not shown are the peaks of C-10 and C-12 which are centered at δ 128.07 and 127.89 ppm, respectively. Peaks 11 and 12, at δ 129.89 and 129.79 ppm, respectively, have not been identified previously. Peak 11 has a shoulder on the low frequency side, as in the case of C-9 of oleic acid. The chemical shift between peak 12 and the main peak of 11 is 0.10 ppm.

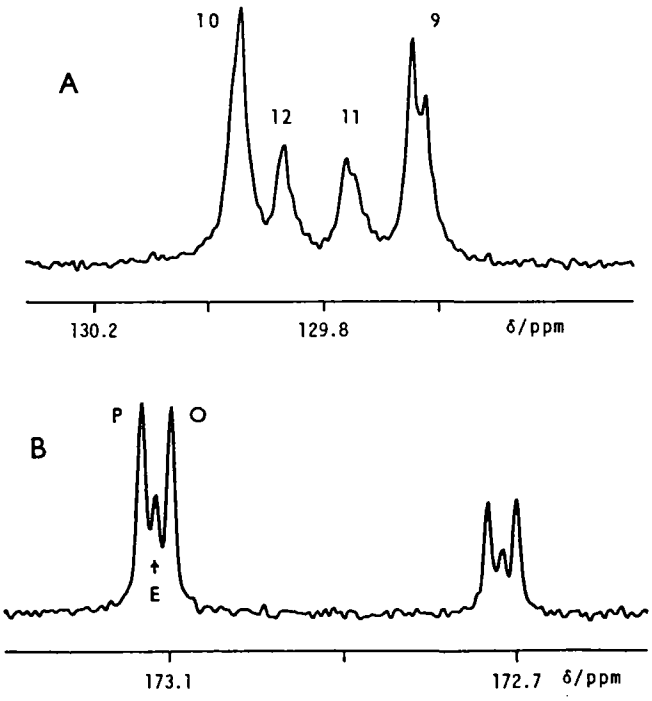

FIG. 3. 25.05 MHz ^{13}C NMR spectra of a mixture of synthetic tripalmitin, triolein and trieicosenoin in the mole ratio 2:2:1, respectively, in chloroform-*d* solution (total conc. 3.0 mol %) at 28 C. A, spectrum of the olefinic carbons. Peaks 9 and 10 belong to C-9 and C-10 of oleic (*cis*-9-monoene), while peaks 11 and 12 belong to C-11 and C-12 of eicosenoic (*cis*-11-monoene). The chemical shift between peak 12 and the main peak of 11 is 0.108 ppm. B, spectrum of the carbonyl carbons. The group of peaks at δ 173.1 ppm pertains to acyl groups at the 1,3-positions, while the group at δ 172.7 ppm pertains to those at the 2-position. Assignments are P, palmitic; O, oleic; E, eicosenoic; assignments are in the same order to the peaks for the 2-position.

TABLE 1

Chemical Shifts of Carbonyl Carbons in Triacylglycerols[a]

Triacylglycerol	δ(1,3)[b]	δ(2)[b]
Tripalmitin (16:0)	173.146	172.735
Tripalmitolein (16:1,[*cis*]-9)	173.114	172.703
Tripetroselinin (18:1,[*cis*]-6)	172.988	172.591
Triolein (18:1,[*cis*]-9)	173.113	172.702
Trielaidin (18:1,[*trans*]-9)	173.121	172.711
Trieicosenoin (20:1,[*cis*]-11)	173.130	172.719
Trierucin (22:1,[*cis*]-13)	173.137	172.726

[a]In chloroform-*d* solution at 28 C, conc. 2.0 mol %; spectrometer operating frequency 25.05 MHz. Chemical shift scale δ in ppm.
[b]δ(1,3) pertains to the acyl groups at the 1,3-positions and δ(2) to that at the 2-position.

as the other peaks and, as such, may be unresolved for a positional isomer, especially when it is of much lower abundance than the methyl oleate. Thus, the *cis*-11-monoene detected in Figures 1 and 2 tentatively is identified as *cis*-vaccenic acid (18:1,[*cis*]-11). This is to be confirmed by isolation of this fatty acid using high resolution capillary column GC. The presence of *cis*-vaccenic acid in palm oil has never been reported (8).

TABLE 2

Chemical Shift Data of the Olefinic Carbons of Monoenes in Triacylglycerols[a]

Triacylglycerol	Δ(1,3)[b]	Δ(2)[b]	δ_m(1,3)[c]
Tripetroselinin	1.613	1.638	127.719
Tripalmitolein	0.292	0.330	129.839
Triolein	0.292	0.330	129.839
Trielaidin	0.298	0.318	130.324
Trieicosenoin	0.108	0.124	129.852
Trierucin	0.038	0.048	129.861

[a]In chloroform-*d* solution at 28 C, conc. 2.0 mol %; spectrometer operating frequency 25.05 MHz. Chemical shift unit in ppm.

[b]Each olefinic carbon appears as two peaks, if resolved, because of the two glycerol positions (7). Δ(1,3) refers to the chemical shift difference in ppm of the two olefinic carbons of the acyl groups at the 1,3-positions, and Δ(2) to that at the 2-position.

[c]δ_m(1,3) refers to the mean of the chemical shifts in ppm of the two olefinic carbons of acyl groups at the 1,3-positions. The corresponding values for the acyl group at the 2-position, δ_m(2), is in every case less than δ_m(1,3) by 0.005 to 0.010 ppm.

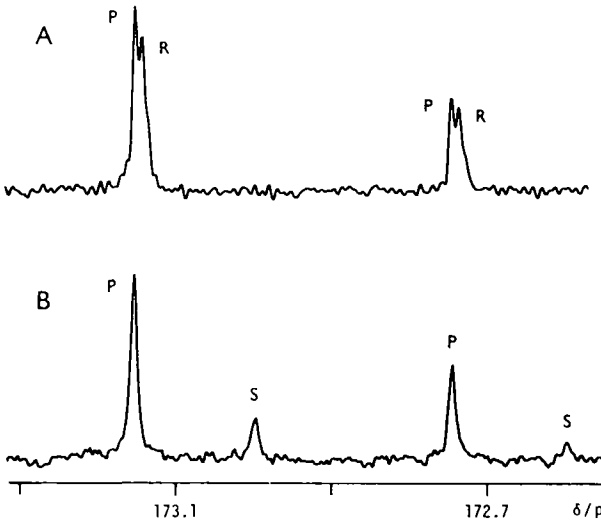

FIG. 4. 25.05 MHz ^{13}C NMR spectra of the carbonyl carbons of mixtures of synthetic triacylglycerols in chloroform-*d* solution (total conc. 2.0 mol %) at 28 C. See Figure 3 for assignment of resonance peaks to the glycerol positions. A, spectrum of the solution of tripalmitin (P) and trierucin (R), showing that the peaks for the carbonyl carbons of the *cis*-13-monoene all appear 0.009 ppm to low frequency of those of saturated tripalmitin. B, spectrum of the solution of tripalmitin (P) and tripetroselinin (S), showing that the peaks for the carbonyl carbons of *cis*-6-monoene appear to low frequency of those of saturated tripalmitin, 0.158 ppm in the case of the 1,3-positions and 0.144 ppm in the case of the 2-position.

The data in Table 1 show that the chemical shift of the carbonyl carbon of a *cis*-monoene in a triacylglycerol depends on the position of the double bond. This may be the result of steric interactions with the glycerol carbons and of the linear electric field effect of the double bond (9). The length of the free end of the acyl chain beyond the double bond does not appear to affect the chemical shift of the carbonyl carbon, as the data for tripalmitolein

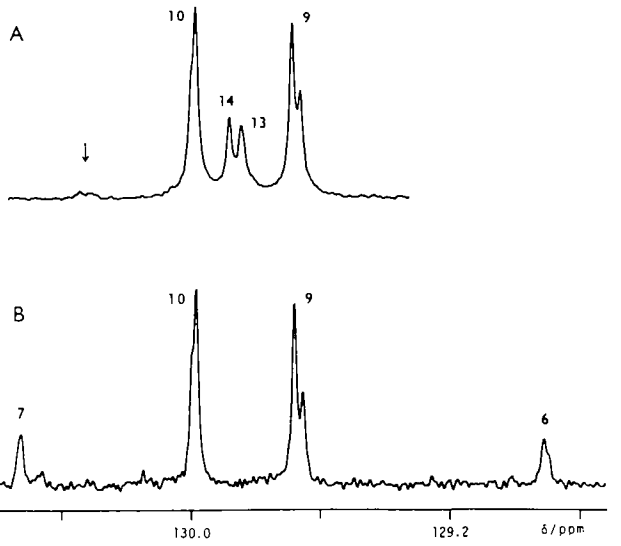

FIG. 5. 25.05 MHz ^{13}C NMR spectra of the olefinic carbons of mixtures of synthetic triacylglycerols in chloroform-*d* solution (total conc. 3.0 mol % in A and 2.0 mol % in B) at 28 C. See Figure 2 for assignment of the peaks to the glycerol positions. A, spectrum of the solution of triolein and trierucin, showing the relative positions of the resonance peaks of the olefinic carbons of *cis*-9- and *cis*-13-monoenes. Peaks 9 and 10 belong to C-9 and C-10 of triolein, while peaks 13 and 14 belong to C-13 and C-14 of trierucin (*cis*-form). The arrow indicates the corresponding peaks, centered at δ 130.34 ppm, of the *trans*-form of trierucin, which is present (ca. 10%) in the sample of trierucin. B, spectrum of the solution of triolein and tripetroselinin, showing the relative positions of the resonance peaks of the olefinic carbons of *cis*-9- and *cis*-6-monoenes. Peaks 9 and 10 pertain to triolein as described above, while peaks 6 and 7 belong to C-6 and C-7 of tripetroselinin.

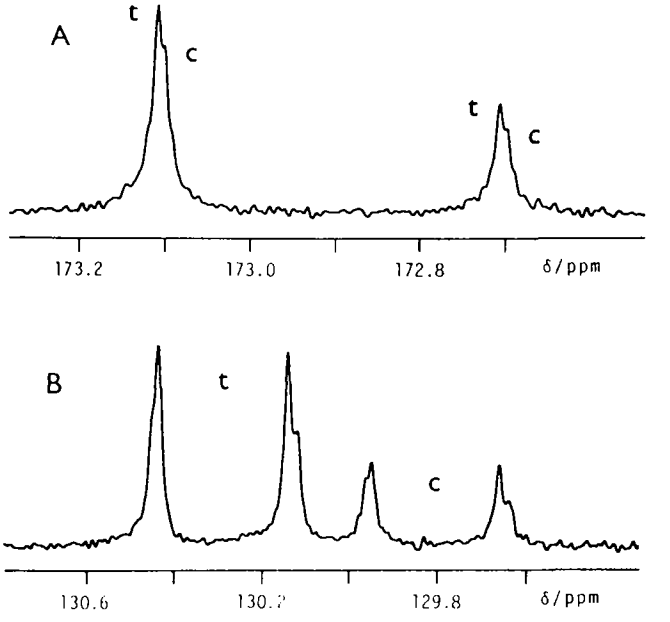

FIG. 6. 25.05 MHz ^{13}C NMR spectra of a mixture of triolein and trielaidin (*trans*-9-monoene), with the latter as the major component (total conc. 2.5 mol %) at 28 C. A, spectrum of the carbonyl carbons, showing that in the *trans*-form (t) each of the resonance peaks appears 0.008 ppm to high frequency of the corresponding peak in the *cis*-form (c). B, spectrum of the olefinic carbons, showing that the peaks for the *trans*-form (t) appear about 0.48 ppm to high frequency of the corresponding peaks in the *cis*-form (c).

(16:1,[*cis*]-9) and triolein (18:1,[*cis*]-9) indicate. When the double bond is nearer to the carbonyl, the resonance of the carbonyl is shifted farther to low frequency; relative to the saturated tripalmitin, the shift to low frequency in the dilute chloroform-*d* solution (ca. 2 mol %) is 0.158 and 0.144 ppm for the 1,3- and 2-glycerol positions, respectively, in the *cis*-6-monoene (tripetroselinin), whereas the shift is only 0.009 ppm for either of the glycerol positions in the *cis*-13-monoene (trierucin), as shown in Figure 4. By extrapolation of the data in Table 1, the shift in the carbonyl carbon resonance from that of the saturated palmitic acid is expected to be negligibly small at low magnetic field in the *cis*-15-monoene and to be undetectable even at high magnetic field in the *cis*-17-monoene.

Table 2 shows that the chemical shift between the two olefinic carbons of a monoene in a triacylglycerol depends on the position of the double bond, being larger when the double bond is nearer to the carbonyl. This may be mainly the result of the linear electric field effect of the head group dipole (9). In a mixture of monoenes, the spectrum of the olefinic carbons of a given monoene always appears inside that of the monoene whose double bond bears a smaller positional number, as shown in Figures 3 and 5. Earlier studies have reported similar chemical shift data for a large number of olefinic fatty acids and esters (10) and of unsaturated fatty acid methyl esters (11,12). Table 2 also shows the position midway between the resonances of the two olefinic carbons of a monoene. By extrapolation, the resonances of the two olefinic carbons are expected to converge at δ 129.87 ppm in the *cis*-17-monoene in dilute chloroform-*d* solution. The chemical shifts of the olefinic carbons of the *trans*-monoene occur at higher frequencies than the corresponding *cis*-monoene, as indicated for trielaidin and triolein in Table 2 and Figure 6, and for trierucin in Figure 5. The chemical shift between the olefinic carbons of a monoene appears to be the same in the *cis*- and the *trans*-isomers. Thus, by reference to or by interpolation of the data in Tables 1 and 2, any monoenoic fatty acid with olefinic positional number up to 15 can be identified by ^{13}C NMR spectroscopy.

This study shows that *cis*-vaccenic acid is present in detectable quantities in the oils of some species of palm, being most abundant in the oil of *E. oleifera*, and that ^{13}C NMR spectroscopy is an effective technique for its detection. Monounsaturated fatty acids and their positional isomers are distinguished easily in the ^{13}C NMR spectra of the carbonyl and the olefinic carbons. The disadvantage of this technique is its insensitivity to the length of the free end of the acyl chain beyond the double bond.

Recently the presence of *cis*-vaccenic acid in mango pulp lipids has been reported (4). It is noteworthy that an appreciable quantity of palmitoleic acid is present together with *cis*-vaccenic acid in the oil of *E. oleifera*, as the former acid is involved in the mechanism of formation of the latter (13). The synthesis of *cis*-vaccenic acid in the seeds of *Sinapis alba* through elongation of preformed palmitoleic acid has been observed recently (14).

REFERENCES

1. Slover, H.T. (1983) *Lipids 18,* 913.
2. Appelqvist, L.-A. (1975) in *Recent Advances in the Chemistry and Biochemistry of Plant Lipids* (Galliard, T., and Mercer, E.L., eds.) pp. 247-286, Academic Press, New York, NY.
3. Nasirullah, Werner, G., and Seher, A. (1984) *Fette Seifen Austrichm. 86,* 264-268.
4. Shibahara, A., Yamamoto, K., Nakayama, T., and Kajimoto, G. (1986) *Lipids 21,* 388-394.
5. Christie, W.W., Brechany, E.Y., Johnson, S.B., and Holman, R.T. (1986) *Lipids 21,* 657-661.
6. Ng, S. (1985) *Lipids 20,* 778-782.
7. Ng, S. (1984) *Lipids 19,* 56-59.
8. Gunstone, F.D. (1967) *Fatty Acids and Their Glycerides,* p. 156, Chapman and Hall, London, England.
9. Batchelor, J.G., Cushley, R.J., and Prestegard, J.H. (1974) *J. Org. Chem. 39,* 1698-1705.
10. Gunstone, F.D., Pollard, M.R., Scrimgeour, C.M., and Vedanayagam, H.S. (1977) *Chem. Phys. Lipids 18,* 115-129.
11. Bus, J., Sies, I., and Lie Ken Jie, M.S.F. (1976) *Chem. Phys. Lipids 17,* 501-518.
12. Bus, J., Sies, I., and Lie Ken Jie, M.S.F. (1977) *Chem. Phys. Lipids 18,* 130-144.
13. Hawke, J.C., and Stumpf, P.K. (1985) *Plant Physiol. 40,* 1023-1032.
14. Mukherjee, K.D. (1986) *Lipids 21,* 347-352.

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Cellular Retinyl Esters and Retinol Among Parenchymal And Stellate Cells in Normal Rat Liver

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[11,12-^{3}H] Retinyl acetate (100 μg/20 μCi/rat) in corn oil was fed by stomach tube to normal male Wistar-Furth rats (~250 g body weight). After 15 days, the contents of retinyl esters and retinol (total retinol) and their ^{3}H-radioactivity were measured in the whole liver, crude parenchymal cells and the purified parenchymal cells, employing differential centrifugation, centrifugal elutriation and high performance liquid chromatography (HPLC) techniques. Of the total liver retinol (nmol/g liver), the crude parenchymal cells had nearly 90%, whereas the purified parenchymal cells had only 21% based on HPLC analysis. Furthermore, of the total liver retinol radioactivity (dpm/g liver) the crude parenchymal cell fraction had 85%, while the purified parenchymal cell fraction had only 16%. Based on the cell number, the crude parenchymal cell fraction was contaminated by retinoid-rich stellate cells to the extent of 4%. It, therefore, was concluded that the parenchymal cells accounted for 16-21%, whereas the stellate cells contributed 79-84% of total retinol stored in the liver under normal steady-state conditions. It also was calculated that on a per mg basis, stellate cells had 200 times more total retinol than parenchymal cells, whereas on a per cell basis each stellate cell had 74 times more total retinol than a parenchymal cell.

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Liver is the main storage site of retinyl esters and retinol in mammals (1,2). Under normal nutritional conditions, as much as 95% of total retinol of the body is stored in the liver, predominantly as retinyl palmitate in the rat (1,2). Liver accumulates dietary retinoids as retinyl esters from the circulating chylomicron remnants (3-6). Liver also plays a dynamic role in retinoid metabolism generating retinol from the stored retinyl palmitate (7-9) or resynthesizing the esters (2,3,10-12) as well as synthesizing the retinol binding protein, which is essential for the transport of retinol to extrahepatic tissues (13,14). Furthermore, liver has the necessary enzyme systems to oxidize retinol to retinal (15) and retinoic acid (16,17).

Liver is composed of two major classes of cells: the parenchymal cells (PC) and the nonparenchymal cells (NPC). The latter group includes the Kupffer, endothelial and stellate cells. Although it is well known that the liver cells play a major role in several aspects of retinoid metabolism, the distribution of retinoids in various types of liver cells is a subject of great interest and some controversy. Thus, depending on the study and the methodology employed, PC (18-20), Kupffer cells (21,22) or stellate cells (23-25) have been suggested as the major sites of vitamin A storage. Olson and Gunning (26) suggested that hepatic retinoids may be distributed equally between PC and stellate cells. These differences may be due to the lack of reproducible methodology for the isolation of these various cell types in pure form and to inconsistency in the vitamin A status of the animals used in these studies. However, a sophisticated methodology employing a centrifugal elutriation technique now has been developed for the isolation of various liver cells in pure form (24,27,28). Utilizing this technique, this study supports the previous observations and suggestions (29-31) that stellate cells are the major species of liver cells that store the vitamin. It will be demonstrated that liver PC account for only 21% of total retinol stored in the liver, whereas the stellate cells store the rest of the vitamin under normal steady-state conditions. This further is confirmed by the demonstration for the first time that based on the contents of labeled total retinol among these cells, parenchymal cells accounted for 16%, while the stellate cells stored 84% of the vitamin in a steady-state condition.

EXPERIMENTAL

[11,12-^{3}H] Retinyl acetate (specific activity 35 Ci/mmol) was a gift from Hoffmann-La Roche (Nutley, NJ). Betafluor and Hydrofluor scintillation fluids were from National Diagnostics (Somerville, NJ). Collagenase (CLS II) was from Worthington Biochemicals (Freehold, NJ). All other chemicals and reagents were of analytical grade.

Isolation and purification of parenchymal and stellate cell fractions from rats administered with [^{3}H] retinyl acetate. Male albino Wistar-Furth rats (Charles River Breeding Labs, Wilmington, MA) weighing 250 g were used throughout this study. The animals were maintained on normal rat chow (1 μg retinyl acetate per g diet) for at least two weeks before experimentation. Each rat was fed by stomach tube a single 100 μg dose of [11,12-^{3}H] Retinyl acetate (20 μCi) in 1 ml corn oil. The rats were fed ad *libitum* for a further period of 15 days to allow the administered tracer to be fully equilibrated with the endogenous pool and thus reach a steady-state (32). A small lobe of each liver was saved on the 15th day for the determination of total retinyl esters and retinol. The total liver cells were isolated from each rat by our published procedure (33), which was according to that of Krebs et al. (34).

Briefly, the liver was perfused at 37 C initially with Krebs-Henseleit bicarbonate buffer, pH 7.4, without $CaCl_2$ (KRB) at a flow rate of 1.5 ml/min/g for 10 min after which 30 mg Collagenase (CLS II) was added to

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Abbreviations: CPC, crude parenchymal cells; HPLC, high performance liquid chromatography; KRB, Krebs-Henseleit bicarbonate; LDH, lactate dehydrogenase; NPC, nonparenchymal cells; PC, parenchymal cells; PPC, purified parenchymal cells.

100 ml of the KRB buffer, and the perfusion was continued for a further period of 20 min when the liver cells were ready to disperse easily. The whole liver was isolated, finely minced with scissors and suspended in Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 1.5% bovine serum albumin, 20 mM dextrose and 2.5 mM $CaCl_2$ (KHB-BDC buffer). The cell suspension was filtered through a nylon filter (mesh size, 250 μm). The crude parenchymal cells (CPC) were sedimented from the total liver cell suspension by centrifugation at 50 $\times$ *g* for two min, leaving the crude NPC in the supernatant fraction. The CPC thoroughly were washed free of residual collagenase by resuspending in KHB-BDC buffer and resedimenting three times as before.

The final washed CPC (2×10^7 cells) were subjected to two successive centrifugal elutriations (27) in a Beckman J6 M centrifuge, using the standard Sanderson chamber in the JE-6 elutriation rotor revolving at 2500 rpm. The CPC were introduced into the chamber at a flow rate of 11 ml/min, which was increased stepwise with time to 13.5, 20, 23, 25, 38, and 53 ml/min. A volume of 100 ml of KHB-BDC buffer was washed through the cells in the chamber at each flow rate, and the effluent was collected. This elutriation procedure was repeated one more time, and the purified parenchymal cells (PPC) that sedimented in the chamber after the second elutriation step were collected. The liver lobe, CPC and PPC were extracted sequentially with 20 vol of methanol and 20 vol of hexane. All organic solvents contained 5 mg % (w/v) butylated hydroxytoluene. The combined lipid extracts were reduced in volume under a stream of nitrogen and analyzed for their retinyl ester and retinol contents by high performance liquid chromatography (HPLC) and for tritium radioactivity.

Characterization of the liver cells. Both PC and NPC were more than 90% viable as tested by trypan blue dye exclusion. PC satisfied all the criteria of intact, viable and metabolically active hepatocytes defined by Krebs et al. (34). Furthermore, the integrity of the plasma membrane of PC was tested by measuring the leakage of cellular lactate dehydrogenase (LDH) activity and of labeled total retinol into the medium when incubated in the KHB-BDC buffer. Only 5% of the total cellular LDH activity and 5-8% of cellular total retinol radioactivity were released into the medium even after three hr of incubation. The NPC fraction lost only 8% of its total retinol radioactivity under identical conditions. Thus, the various types of liver cells isolated in this study were characterized to be intact, viable and metabolically active. Fluorescent microscopy revealed that the CPC fraction was contaminated by stellate cells to the extent of 4% based on cell number. In contrast, the PPC fraction virtually was devoid of any contamination by stellate cells.

Determination of retinyl ester and retinol by HPLC. HPLC was run in a Beckman Model 346 system equipped with a Beckman Model 165 detector, auto sampler and model 450 data system. The sample was applied to a Zorbax TMS column (4.6 $\times$ 15 cm; E.I. Dupont de Nemours & Co., Wilmington, DE) using acetonitrile: water (85:15, v/v) with a flow rate of 1.2 ml/min. Authentic retinol, retinyl acetate and retinyl palmitate were eluted as sharp peaks at 2.2, 2.5 and 9.1 min, respectively. Recoveries of known quantities of all these retinoids, when added to the cell fractions before extraction, were more than 95% using this procedure. The detection limit of this method was 5-10 ng for each retinoid.

RESULTS AND DISCUSSION

Table 1 shows the relative levels of retinyl esters and retinol as well as the relative distribution of total retinol radioactivity in the whole liver, CPC, PPC and the stellate cells 15 days after oral administration of (11,12-^{3}H)retinyl acetate (100 μg/20 μCi/rat) to normal rats. Based on HPLC analysis, the normal rat liver had 679 nmol of total retinol per g wet wt under steady state conditions. Significantly, the CPC fraction had 611 nmol/g (data not shown), whereas the PPC fraction had 144 nmol/g (Table 1). Thus, the CPC had nearly 90%, while the PPC had only 21% of the liver total retinol. These data indicate that even though the crude parenchymal fraction contained most of the liver total retinol as observed by others (18,19) and in our preliminary report (20), purification by centrifugal elutriation led to the retention of only

TABLE 1

Relative Level of Total Retinol in Parenchymal and Stellate Cells

Cell type	Retinyl esters plus retinol[a] (nmol/g wet wt)	As % of total liver	Radioactivity[b] dpm $\times 10^{-5}$/g wet wt	As % of total liver
Total liver	679 ± 117	100	3.26 ± 0.37	100
Parenchymal	144 ± 29	21	0.53 ± 0.07	16
Stellate[c]	535 ± 91	79	2.73 ± 0.04	84

[a]Each value is the mean of six animals ± SE. 86.5% of the total amount was due to retinyl palmitate, 8.6% due to the sum of retinyl stearate and oleate and 4.9% due to retinol.
[b]Each value is the mean of four animals ± SE.
[c]These values were calculated by subtracting the values of parenchymal cells from the total liver values.

TABLE 2

Relative Concentration of Total Retinol in Parenchymal and Stellate Cells

	Parenchymal cells	Stellate cells	S/P cells
Total retinyl esters plus retinol[a] (nmol/g liver)	144	535	3.7
Mass of cell type (37) (mg/g liver)	810	15	0.02
No of cells $\times 10^{-6}$/g liver (38)	100	5	0.05
Retinyl esters plus retinol concentration			
nmoles/mg	0.18	35.67	200
nmoles/10^6 cells	1.44	107	74

[a]Values were taken from Table 1.

a small percentage of total retinol of the liver. This would mean that most of the vitamin recovered in the CPC fraction must have been due to the contaminating stellate cells. This is further confirmed by our finding of the relative distribution of total retinol radioactivity among various liver cells (Table 1). It was found that under identical conditions, the total retinol label in the whole liver was 3.26×10^5 dpm/g of which only 0.53×10^5 dpm/g were recovered in PPC. Thus, PPC accounted for only 16% while the other 84% of the liver total retinol label was due to the NPC. Of the NPC population, endothelial and Kupffer cells contain negligible amounts of the vitamin (30). Therefore, the only other NPC type that is the major storage site of liver vitamin A under steady-state conditions in normal rats is the stellate cell. It also is clear that PC contribute to the extent of 16-21% of the liver total retinol under identical conditions. This distribution pattern probably is most dependent upon the vitamin A status of the animals as well as their age. For example, PC seemed to play a major role in the uptake and storage in a vitamin A-deficient state (35,36) while stellate cells seemed to be the major storage site when the liver total retinol concentration was varied over a three-fold range from 140-390 μg/g (27). The relative roles of various liver cells in hypervitaminosis A remain to be explored.

As illustrated in Table 2, PC constitute 81%, whereas the stellate cells possibly account for only 1.5% of total liver mass (37). Therefore, one can calculate the concentration of total retinol on a wet weight basis of each cell type. Thus, the stellate cells contain 35.67 nmol/mg while the PC have 0.18 nmol/mg, a concentration difference of 200-fold. Each g wet weight of the liver contains 100×10^6 PC and 5×10^6 stellate cells (38). Thus, when the concentration of total retinol is calculated based on cell number, stellate cells contain 107 nmol/10^6, whereas PC have 1.44 nmol/10^6, which amounts to 74 times more total retinol per stellate cell than per PC.

REFERENCES

1. Moore, T. (1957) in *Vitamin A*, pp. 208–210, Elsevier, Amsterdam, The Netherlands.
2. Goodman, D.S., and Blaner, W.S. (1984) in *The Retinoids* (Sporn, M.B., Roberts, A.B., and Goodman, D.S., eds.) Vol. 2, pp. 1–39, Academic Press, New York, NY.
3. Goodman, D.S., Huang, H.S., and Shiratori, T. (1965) *J. Lipid Res. 6*, 390–396.
4. Lawrence, C.W., Crain, F.D., Lotspeich, F.J., and Krause, R.F. (1966) *J. Lipid Res. 7*, 226–229.
5. Goodman, D.S., Stein, O., Halperin, G., and Stein, Y. (1983) *Biochim. Biophys. Acta 750*, 223–230.
6. Larsson, B., and Nilsson, A. (1983) *Biochim. Biophys. Acta 751*, 474–478.
7. Zachman, R.D., and Olson, J.A. (1963) *J. Biol. Chem. 238*, 541–546.
8. Zachman, R.D., and Olson, J.A. (1965) *J. Lipid Res. 6*, 27–32.
9. Ross, A.C. (1982) *J. Biol. Chem. 257*, 2453–2459.
10. Harrison, E.H., Smith, J.E., and Goodman, D.S. (1979) *J. Lipid Res. 20*, 760–771.
11. Prystowsky, J.H., Smith, J.E., and Goodman, D.S. (1981) *J. Biol. Chem. 256*, 4498–4503.
12. Blaner, W.S., Prystowsky, J.H., Smith, J.E., and Goodman, D.S. (1984) *Biochim. Biophys. Acta 794*, 419–427.
13. Smith, J.E., Borek, C., and Goodman, D.S. (1978) *Cell 15*, 865–873.
14. Goodman, D.S. (1984) in *The Retinoids* (Sporn, M.B., Roberts, A.B., and Goodman, D.S., eds.) Vol. 2, pp. 41–88, Academic Press, New York, NY.
15. Zachman, R.D., and Olson, J.A. (1961) *J. Biol. Chem. 236*, 2309–2312.
16. Dimitrovsky, A.A. (1961) *Biokhimiya 26*, 126.
17. Bamji, M.S., Mahadevan, S., Lakshmanan, M.R., and Murthy, S.K. (1962) *Nature London 196*, 672–673.
18. Peterson, P.A., Rask, L., Andersson, L., Kamwendo, F., and Pertoft, H. (1973) *J. Biol. Chem. 248*, 4009–4022.
19. Linder, M.C., Anderson, G.H., and Ascarelli, I. (1971) *J. Biol. Chem. 246*, 5538–5540.
20. Sundaresan, P.R., and Lakshmanan, M.R. (1984) *Fed. Proc. 43*, 787.
21. Rodahl, K. (1950) *J. Nutr. 41*, 399–421.
22. Lane, B.P. (1968) *Am. J. Pathol. 52*, 591–594.
23. Wake, K. (1980) *Int. Rev. Cytol. 66*, 303–353.
24. Knook, D.L., Seffellaar, A.M., and de Leeuw, A.M. (1982) *Exp. Cell Res. 139*, 468–471.

25. Hirosawa, K., and Yamada, E. (1973) *J. Electron Microsc. 22*, 337–346.
26. Olson, J.A., and Gunning, G. (1983) *J. Nutr. 113*, 2184–2191.
27. Batres, R.O., and Olson, J.A. (1987) *J. Nutr. 117*, 77–83.
28. de Leeuw, A.M., McCarthy, S.P., Geerts, A., and Knook, D.L. (1984) *Hepatology 4*, 392–403.
29. Blomhoff, R., Smedsrod, B., Eskild, W., Granum, P.E., and Berg, T. (1984) *Exp. Cell Res. 150*, 194–204.
30. de Leeuw, A.M., Brouwer, A., Van den Berg, K.J., and Knook, D.L. (1983) *Proc. Nutr. Soc. 42*, 8A.
31. Blomhoff, R., Rasmussen, M., Norum, K.R., Berg, T., Blaner, W.S., Kato, M., Mertz, J.R., Goodman, D.S., Eriksson, U., and Peterson, P.A. (1985) *J. Biol. Chem. 260*, 13560–13565.
32. Sundaresan, P.R. (1977) *J. Nutr. 107*, 70–78.
33. Lakshmanan, M.R., Muesing, R.A., and La Rosa, J.C. (1981) *J. Biol. Chem. 256*, 3037–3043.
34. Krebs, H.A., Cornell, N.W., Lund, P., and Hems, R. (1974) in *Alfred Benzon Symposium* (Lundquist, F., and Tygstrup, N., eds.) Vol. VI, pp. 726–750, Munksgaard, Copenhagen, Denmark.
35. Blomhoff, R., Helgerud, P., Rasmussen, M., Berg, T., and Norum, K.R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7926–7930.
36. McKenna, M.C., and Bieri, J.G. (1982) *Am. J. Clin. Nutr. 35*, 1010–1017.
37. Blouin, A., Bolender, P., and Weibel, E.R. (1977) *J. Cell Biol. 72*, 442–455.
38. Knook, D.L., and Sleyster, E.C. (1976) *Exp. Cell Res. 99*, 444–449.

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A Highly Active Soluble Diacylglycerol Synthesizing System from Developing Rapeseed, *Brassica napus* L.

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The subcellular distribution of the enzymes of triacylglycerol biosynthesis has been studied in developing oilseed rape. All in vitro enzymatic activities from oleoyl-CoA to triacylglycerol were sufficient to account for the known rate of oleate deposition in triacylglycerol in vivo. The enzymatic activities from oleoyl-CoA to diacylglycerol preferentially were localized in a 150,000 g supernatant fraction, while the diacylglycerol acyl-transferase mostly was associated with the microsomal (20,000 g pellet and 150,000 g pellet) and oil-body fractions. The soluble (150,000 g supernatant) fraction rapidly incorporated oleate from [1-^{14}C]oleoyl-CoA into diacylglycerol with rates of 40 nm min^{-1} g^{-1} FW at 20 μM oleoyl-CoA. The pH optimum was 7.5–9.0, and normal saturation kinetics were seen with oleoyl-CoA; the $S_{0.5}$ was about 32 μM. Exogenous acyl acceptors, such as glycerol 3-phosphate, lysophosphatidic acid and lysophosphatidylcholine stimulated oleate incorporation into diacylglycerol. The detergents Triton X-100 and sodium cholate inhibited diacylglycerol formation at concentrations in the region of their critical micellar concentration, while n-octyl-β,D-glyco-pyranoside had no effect, even at high concentration. The significance of these findings for the mechanism of oil-body formation in developing oilseeds is discussed.
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Triacylglycerols are the major form in which lipid is stored in many plant tissues, especially the oilseeds. Despite the evident economic usefulness of oilseed triacylglycerols and the desirability of manipulating their acyl composition to produce improved crop varieties, many aspects of their deposition in developing seeds remain obscure. In particular, the subcellular localization of the enzymes involved in triacylglycerol biosynthesis and the mechanism of oil-body formation yet have to be resolved satisfactorily. Triacylglycerols are synthesized by the progressive acylation of glycerol 3-phosphate by three acyltransferases while the phosphate moiety is removed by a phosphatase. Together, these reactions constitute the Kennedy pathway (1) for triacylglycerol biosynthesis.

Heinz et al. have reported that pea and spinach leaves contain one soluble (chloroplast stroma) (2–4) and one membrane-bound (microsomal) (5) glycerol 3-phosphate acyltransferase (GP-AT) plus two membrane-bound (chloroplast envelope and microsomal) (6,7) lysophosphatidic acid acyltransferases (LPA-AT). It also has been reported that spinach leaves contain a membrane-bound (chloroplast envelope) diacylglycerol acyltransferase (DG-AT) (8,9). In the case of developing oilseeds, both a soluble (10) and a microsomal (11) GP-AT have been found, and there are several reports of microsomal DG-AT activity (12–14), one of which shows that the enzyme in maize and castor bean seeds derives from endoplasmic reticulum rather than plastid envelope membranes (14). Purified oil-body fractions from crambe (15) and rapeseed (16) have been shown to incorporate oleoyl-CoA into triacylglycerols and, therefore, must contain all of the above acyltransferases, i.e. GP-AT, LPA-AT and DG-AT. Similar activities also have been reported in soluble extracts of developing rapeseed (16) but the soluble and oil-body acyltransferases remain to be characterized.

The determination of the subcellular localization of the enzymes of triacylglycerol biosynthesis will be important in shedding light on the ontogeny of oil-bodies (spherosomes) in developing oilseeds. This currently is the subject of much controversy with some authors favoring the development of oil-bodies from endoplasmic reticulum (17–19), while others propose several alternative mechanisms (20–24). Quite apart from the possibility that oil-bodies may be formed from extra-plastid membrane systems such as endoplasmic reticulum it also is possible that they may be derived directly from plastid fatty acids in the following manner. Plastids are the major and possibly the unique site of acyl-CoA biosynthesis (25). Soluble cytoplasmic GP-AT, LPA-AT and phosphatidic acid (PA) phosphatase could interact with an oil-body DG-AT to synthesize triacylglycerols directly from plastidic oleoyl-CoA without recourse to endoplasmic reticulum enzymes. Such a hypothesis would be consistent with the cytoplasmic origin of oil-bodies as proposed by Bergfeld et al. (22).

In order to test the feasibility of this hypothesis, the soluble DG-synthesizing activities of developing rapeseed have been investigated and compared with those of oil-body and microsomal fractions. The data show that these soluble enzymes are capable of making a substantial contribution to total triacylglycerol biosynthesis in developing oilseeds.

MATERIALS AND METHODS

Materials. Rapeseed plants (*Brassica napus* L., var. Jet neuf) were grown in glasshouses at the Durham University Botanical Gardens. Developing seeds were harvested four to six weeks after flowering. This was the stage of maximum triacylglycerol deposition. These seeds contained virtually no erucic acid (<0.5% total fatty acid).

Radiochemicals were purchased from Amersham International (U.K.). Reagents and cofactors such as CoASH, oleoyl-CoA, reduced nicotinamide adenine dinucleotide (NADH), adenosine triphosphate (ATP) and lysophosphatidylcholines were obtained from Sigma (U.K.). Precoated TLC plates were from Anachem (U.K.). Analytical grade solvents were used throughout. Reference lipids used in chromatography were from Sigma.

Abbreviations: ATP, adenosine triphosphate; CMC, critical micellar concentration; DG, diacylglycerol; DG-AT, diacylglycerol acyltransferase; DTT, dithioltreitol; EDTA, ethylenediamine tetraacetic acid; FW, freshweight; G3P, glycerol 3-phosphate; GP-AT, glycerol 3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPA-AT, lysophosphatidic acid acyltransferase; LPC, lysophosphatidylcholine; MG, monoacylglycerol; NADH, reduced nicotinamide adenine dinucleotide; PA, phosphatidic acid; PC, phosphatidylcholine.

Isolation of subcellular fractions. Intact embryos were dissected carefully out of the seeds and placed in a small volume of isolation buffer at 0 C. The isolation buffer consisted of 50 mM HEPES-KOH, pH 7.4, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithioltreitol (DTT), and 0.5 M sucrose. The embryos gently were ground in a mortar and filtered through two layers of Miracloth, prewetted with isolation buffer. This crude homogenate was centrifuged at 12,000 g for 15 min to yield crude oil-body (O_1), pellet (P_1) and supernatant (S_1) fractions. The crude oil-body fraction was purified by further centrifugation as described by Cao et al. (14). The supernatant was made up to 50 mM $MgCl_2$ and centrifuged at 20,000 g for 20 min. The resulting pellet (P_2), which was light-green, was the crude microsomal fraction. The supernatant (S_2) from this step was centrifuged at 50,000 g for 30 min and finally at 150,000 g for 60 min. The final colorless pellet (P_4) was retained for assays, and the final clear supernatant (S_4) formed the soluble protein fraction.

Incubations. A rapid small-scale assay system was developed to deal with the large number of samples used in this study. Incubations normally were performed in duplicate except in which "internal duplicates" already were part of the protocol, e.g. a time course or a protein curve. The basic incubation medium consisted of 0.1 M potassium phosphate, pH 7.2, 10 mM $MgCl_2$, to which the appropriate protein fraction and 0.5 K Bq [1-^{14}C]-oleoyl-CoA was added at a final concentration of 20 μM unless otherwise stated, to give a total volume of 55 μl. Lipidic substrates (G3P, DG, MG, LPA, PA, LPC, PC) were added as sonicated dispersions as described elsewhere (26). Incubations were carried out either in open glass tubes or in 1.8 ml-capacity Eppendorf tubes. The latter had the advantage of allowing for the simultaneous processing of larger numbers of samples. The results of incubations and lipid extractions in glass tubes and Eppendorf tubes were identical.

Incubations were performed at 25 C in a shaking water bath. Reactions were terminated by the addition of 110 μl isopropanol, followed by heating at 80 C for five min. Either 1.1 ml chloroform/methanol (2:1, v/v) or dichloromethane/methanol (2:1, v/v) was added to each tube, which was vortexed before the addition of 220 μl of an aqueous solution of 0.1% acetic acid containing 0.7% NaCl. The tubes were vortexed thoroughly and centrifuged at 1,000 g to ensure adequate phase separation. All water-soluble constituents such as acyl-CoAs partioned into the upper, aqueous phase. The lower phase, containing the chloroform-soluble lipids, was recovered for analysis.

Analytical procedures. Total lipids were fractionated by chromatography on precoated 20 × 5 cm Silica Gel G plates. Neutral lipids were resolved in a hexane/diethylether/acetic acid (70:30:1, v/v/v) system following a preliminary double development with diethylether 3 cm above the origin in order to resolve monoacylglycerols from polar lipids. Plates were run with the authentic reference compounds, monoacylglycerol, diacylglycerol, unesterified fatty acid and triacylglycerol. Polar lipids were resolved in chloroform/methanol/water (65:25:4 or 65:35:5, v/v/v) or in chloroform/pyridine/formic acid (50:30:7, v/v/v) in the presence of authentic PC, LPC, PA and LPA. The latter system gave an excellent separation of PA without the streaking often observed in other systems (27) but did not resolve PC and LPC very well. Therefore, sometimes a double development was carried out with plates being run for 10 cm in the former systems, followed by a second running in the pyridine system. Normally, four lanes were run on each 20 × 5 cm thin layer plate. Radioactivity on thin layer plates was quantitatively detected using an Isomess RITA IM 3000 (Isotopen Messgeräte GmBH, Essen, West Germany) automatic plate scanner interfaced to an Apple IIe computer. The scanner was controlled, and data were evaluated using Nuclear Interface software routines (Nuclear Interface, Münster, West Germany). All data from the scanner were double checked by the scintillation counting of representative lipid bands from TLC plates and in this way, reliable quantitative values for radioactivity incorporation were obtained. Lipids were identified on the basis of cochromatography with reference compounds in at least two different TLC systems and also by the use of specific spray reagents. For routine localization of lipid bands, nonspecific staining with either iodine vapor or dichlorofluorescein (0.5% in methanol) was employed. Protein concentrations were assayed according to the method of Markwell et al. (28).

RESULTS AND DISCUSSION

The biosynthetic capacities of the three major subcellular fractions involved in acyl-CoA metabolism in developing rapeseed are compared in Table 1. The most striking difference between the subcellular fractions is the very low levels of phosphatidic acid (PA) and TG in the supernatant fraction as compared with the microsomal and oil-body fractions. The labeling pattern of the microsomal fraction was the same whether the 20,000 g pellet (P_2) or the 150,000 g pellet (P_4) was used for incubations. The oil-body and microsomal fractions utilized the exogenous acyl acceptors lysophosphatidic acid (LPA) and lysophosphatidylcholine (LPC) to form phosphatidic acid and phosphatidylcholine (PC), respectively, in the presence of [^{14}C]oleoyl-CoA. In contrast, the soluble fraction incorporated [^{14}C]oleoyl-CoA mostly into DG irrespective of the presence of lysophospholipid exogenous acyl acceptors (see also Fig. 4). These results are consistent with those of several recent reports on the biosynthetic capacities of subcellular fractions from developing mustard seed (29) and developing rapeseed (16).

The relative activities of the various subcellular fractions in the incorporation of oleoyl-CoA into total lipids show that the total microsomal fraction, which up to now has been regarded as the major source of TG-synthesizing activity in developing oilseeds in fact had the lowest biosynthetic activity (Table 1). The oil-body fraction was about 20% more active than the microsomal fraction but by far the most active subcellular fraction was 150,000 g supernatant (or soluble protein) fraction. The latter fraction showed two to three times more activity than either the microsomal or oil-body fractions. These differences were even more marked when oleoyl-CoA incorporation into DG was considered. The total microsomal and oil-body fractions showed approximately equal capacities for DG synthesis but the soluble fraction was capable of five- to seven-fold more DG synthesis than either of them. This high capacity of the soluble fraction for DG synthesis as

TABLE 1

The Incorporation of [1-^{14}C]oleoyl-CoA by Different Subcellular Fractions of Rapeseed

Subcellular fraction	Exogenous acylacceptor (20 μM)	Incorporation of oleate into acyl lipids (nmol min^{-1} g^{-1} FW)[a]						
		MG	DG	TG	LPA + LPC	PA	PC	Total incorporation into lipids
Microsomes	G3P	0.2	6.4	4.9	6.9	0.2	5.1	24.5
	LPC[b]	0.5	9.8	7.4	2.5	0.1	27.0	49.4
	LPA[b]	0.5	5.4	2.7	6.8	22.7	4.5	45.5
Oil-bodies	G3P	0.3	7.9	7.0	3.2	0.2	5.7	30.3
	LPC	0.5	10.9	9.3	2.6	0.5	21.8	55.1
	LPA	0.5	7.7	4.6	5.2	20.6	11.2	51.8
Soluble fraction	G3P	3.6	40.0	0.7	10.9	0.7	14.5	73.1
	LPC	2.5	35.8	0.6	6.4	0.6	16.0	63.3
	LPA	5.7	47.3	0.8	14.2	3.8	14.2	94.5

DG, diacylglycerol; LPA + LPC, lysophosphatidic acid + lysophosphatidylcholine; MG, monoacylglycerol; PA, phosphatidic acid; PC phosphatidylcholine; TG, triacylglycerol.

[a]Microsomes, oil-bodies and a soluble fraction from developing rapeseed were incubated for 2–10 min with 20 μM [1-^{14}C]oleoyl-CoA (0.05 μCi) in the presence of 20 μM of the three acylacceptors listed above as described in the Materials and Methods section. The figure for total oleate incorporation per subcellular fraction was calculated by the following equation:

$$\text{Total incorporation rate} = \text{incorporation rate per assay} \times \frac{\text{total protein volume per fraction}}{\text{protein volume per assay}}$$

[b]LPC, 1-palmitoyl lysophosphatidylcholine; LPA, 1-oleoyl lysophosphatidylcholine.

compared with the microcomal or oil-body fractions repeatedly was demonstrated using different seed batches and with a different variety of rapeseed (Mikado). On a protein basis, the DG-synthesizing capacities of the microsomal and soluble fractions were about equal but this cannot be a valid estimate of specific activity because, as shown by gel electrophoresis, the soluble fraction contained large amounts of the nonenzymatic seed storage proteins cruciferin and napin.

The maximal rate of oleate accumulation in developing rapeseed in vivo occurred between 28 and 35 days after flowering and was of the order of 2–3 mg oleate formed per day per gFW seed. The seeds used in this study were harvested at this developmental stage. The maximal rate of oleate incorporation into DG by the soluble protein fraction of such seeds at 20 μM oleoyl-CoA was 40 nmol min^{-1} g^{-1} FW, which corresponds to 0.7 mg oleate incorporated hr^{-1}, g^{-1} FW. Over a 12-hr day, this would give rise to an oleate incorporation rate in excess of 8 mg g^{-1} FW, which easily would account for the known rate of oleate accumulation in vivo. The much greater (four-fold) rate of oleate incorporation in vitro compared with the known rate of oleate deposition in vivo suggests that the enzymatic activities from oleoyl-CoA to DG are not rate limiting to TG synthesis.

The rate-limiting factor may be the final enzyme in the Kennedy pathway (1), i.e. the DG acyltransferase that converts DG into TG, although it has been suggested that in some developing oilseeds this enzyme too may have more than sufficient activity to account for the known rate of TG synthesis (14). In this study, the maximal rate of oleate incorporation into TG by microsomal and oil-body fractions of developing rapeseed (the soluble fraction had a much lower rate of oleate incorporation into TG) was about 30 nmol min^{-1} g^{-1} FW, albeit at high (120 μM) oleoyl CoA concentration. This translates to 0.5 mg oleate incorporated into TG hr^{-1} g^{-1} FW, which is far in excess of the known rate in vivo of 2–3 mg dy^{-1} g^{-1} FW. Even at much lower oleoyl-CoA concentrations (20 μM), the rate of oleate incorporation into TG was 0.11 mg hr^{-1} g^{-1} FW, which is of the same order as the in vivo rate. Therefore, it may be that rather than DG-AT activity it is the supply of oleoyl-CoA itself that is the ultimate rate-limiting factor in TG deposition in developing rapeseed.

Because the major lipid biosynthetic activity of the rapeseed soluble protein fraction was DG formation, it was decided to study this activity more closely. The formation of DG from [^{14}C]oleoyl-CoA by the soluble fraction was time-dependent and also was dependent on the amount of added protein, as shown in Figure 1A. At the lowest protein concentrations, the reaction was linear for up to 60 min but at higher protein concentrations the linear phase lasted only for about 10 min. There was no DG synthesis in the presence of boiled protein fractions. The major product of [^{14}C]oleoyl-CoA incubations with the soluble fraction was DG but with time there also was some accumulation of PC, LPA and MG. If [^{14}C]G3P were used as an acyl acceptor in the presence of unlabeled oleoyl-CoA, a slightly different labeling pattern was observed (Fig. 1B). DG still was the major product at the earlier incubation times but after one hr there was a steady increase in the formation of LPA and PC.

These results show that the soluble protein fraction of developing rapeseed contained very active G3P and LPA acyl transferases. Because DG was a major product and PA did not accumulate during incubations there also must be a soluble PA phosphatase in developing rapeseed. This enzyme up to now only has been detected as a membrane-bound activity associated with chloroplast

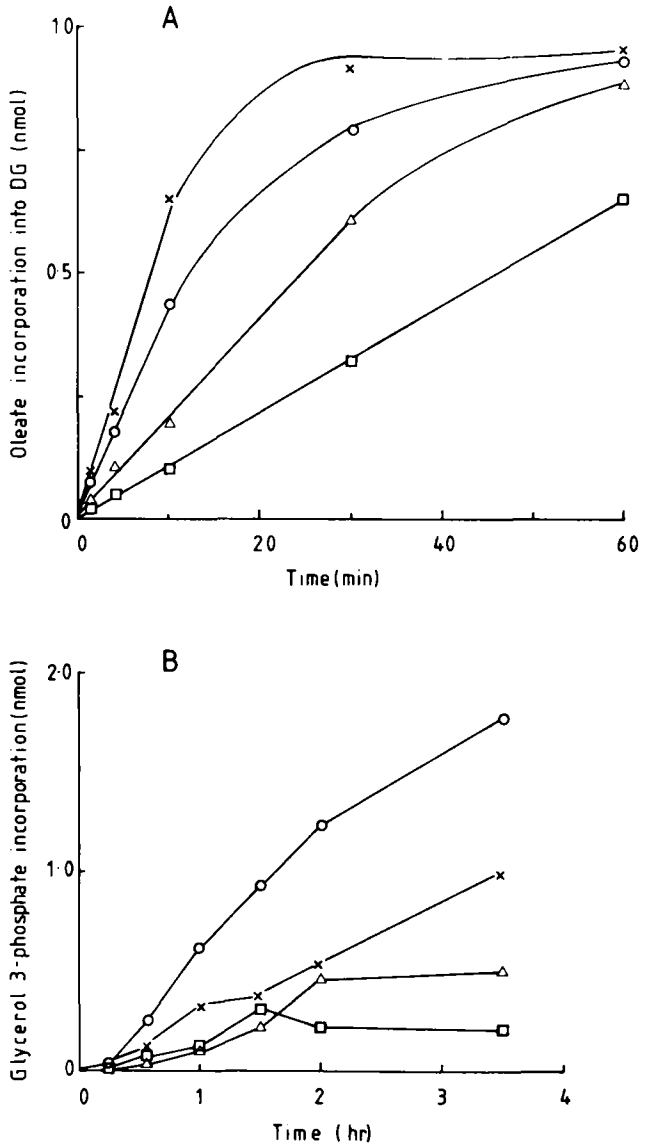

FIG. 1. Time course of the incorporation of oleate into diacylglycerol and other lipids following incubation of (A) various concentrations of a soluble protein fraction from developing rapeseed with [1-^{14}C]-oleoyl-CoA. Incubations contained 20 μM [1-^{14}C]oleoyl-CoA and 35 μM glycerol 3-phosphate in a total volume of 55 μl. Soluble fraction protein was added to each assay as follows: 20 μl, ×—×; 10 μl, ○—○; 5 μl, △—△; 2 μl, □—. Total volume of the soluble fraction was 4 ml from an original 500 mg FW peeled rapeseed. (B) The soluble protein fraction (10 μl) with [U-^{14}C]glycerol 3-phosphate. Incubations contained 100 μM oleoyl-CoA and 150 μM [U-^{14}C]glycerol 3-phosphate. Total lipid, ○—○; lysophosphatidic acid, ×—×; phosphatidylcholine, △—△; diacylglycerol, □—□.

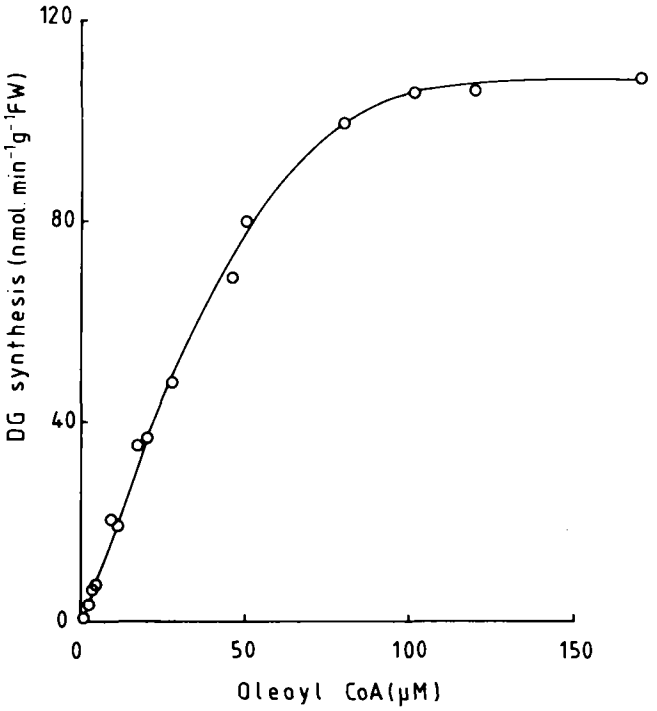

FIG. 2. Effect of oleoyl-CoA concentration on the incorporation of oleate from [1-^{14}C]oleoyl-CoA into diacylglycerol by a soluble fraction from developing rapeseed. Assays were for 20 min and contained 100 μM glycerol 3-phosphate, 20 μM [1-^{14}C]oleoyl-CoA, 10 μl soluble protein, 10 mM $MgCl_2$, 0.1 M potassium phosphate buffer, pH 7.2, in a total volume of 55 μl.

envelopes in leaf tissue (30). The accumulation of [^{14}C]PC following incubation with [^{14}C]G3P strongly suggests that there is a transfer of glycerol moieties between DG and PC. Because [^{14}C]PC also accumulated, albeit to a lesser extent, following incubation with [^{14}C]oleoyl-CoA it is possible that the entire DG molecule may be converted to PC by means of CDP-choline:1,2-DG cholinephosphotransferase, as suggested by Slack et al. (31,32).

DG formation from [^{14}C]oleoyl-CoA by the soluble protein fraction showed a relatively broad optimal pH range with maximum activity pH between 7.5–9.0. This pH optimum is slightly higher than most other TG-synthesizing enzymes studied to date, which typically show pH optima of 7–8 (3,11,12,14,33). DG formation exhibited normal saturation kinetics with respect to oleoyl-CoA with an $S_{0.5}$ of about 32 μM oleoyl-CoA (Fig. 2). Since at least three enzymatic activities were being measured here, it is not possible to derive further kinetic constants for any of the individual components. This must await the further purification of these enzymes, which now is underway in this laboratory.

The effect of various exogenous acyl acceptors on the incorporation of [^{14}C]oleoyl-CoA into DG is shown in Figure 3. DG formation was stimulated in the presence of 20 μM G3P and LPA. Since the oleoyl-CoA concentration in these incubations was 10 μM, it appears that oleoyl-CoA:acyl acceptor ratios in the region of 1:1 are optimal for DG formation, rather than their absolute concentrations over the range tested. At concentrations higher than 20 μM, G3P caused up to 50% inhibition compared to the maximal rate, while higher concentrations of LPA inhibited by as much as 80%. The strong inhibition of DG formation by high concentrations of LPA partially may result from a detergent effect. The addition of exogenous DG, MG and PA did not stimulate DG formation at any concentration but rather caused a 20–50% inhibition of the activity. In contrast to the other acyl acceptors, LPC additions resulted in 20% stimulation of DG formation even at LPC concentrations in excess of 200 μM. In all of the incubations shown in Figure 3, DG

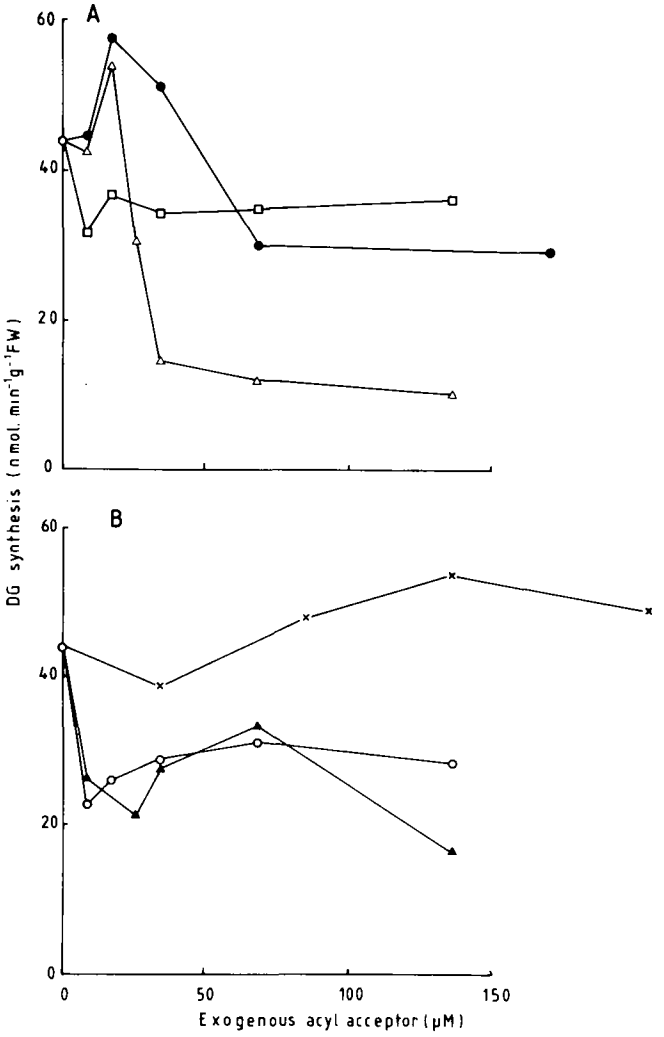

FIG. 3. Effect of different concentrations of various exogenous acyl acceptors on the incorporation of oleate from [1-^{14}C]oleoyl-CoA (10 μM) into diacylglycerol by a soluble fraction from developing rapeseed. Glycerol 3-phosphate, ●—●; lysophosphatidic acid (monooleoyl glycerol 3-phosphate), △—△; diacylglycerol (diolein), □—□; phosphatidic acid (dioleoylglycerol 3-phosphate), ▲—▲; monoacylglycerol (monoolein), ○—○; lysophosphatidic choline (monopalmitoyl glycerol 3-phosphocholine), ×—×. All incubations were for 20 min as described in Methods.

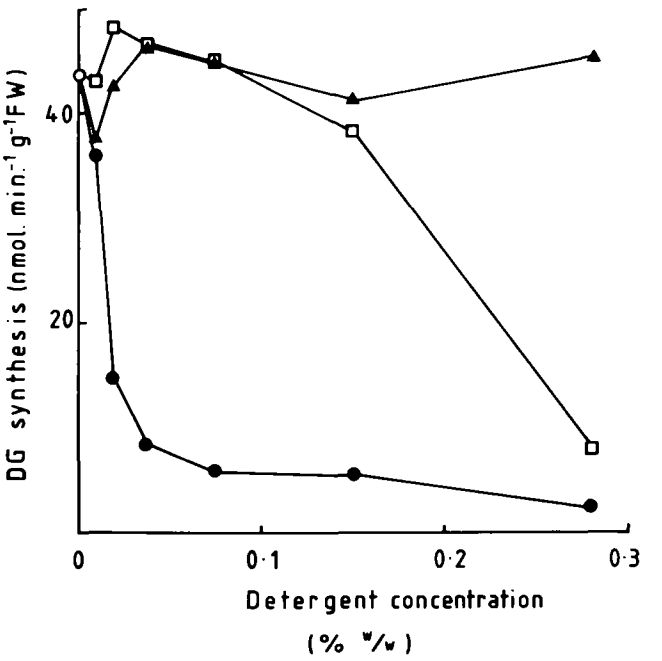

FIG. 4. Effect of different concentrations of various detergents on the incorporation of oleate from [1-^{14}C]oleoyl-CoA into diacylglycerol by a soluble fraction from developing rapeseed. *n*-Octyl β-D glycopyranoside, ▲—▲; Triton X-100, ○—○; sodium cholate, □—□. All incubations were for 20 min as described in Methods.

was overwhelmingly the major reaction product, constituting between 50–70% of total ^{14}C-labeled lipid. The proportion of ^{14}C-label in DG was slightly higher in the presence of G3P (60–70%) compared to the other acyl acceptors (50–60%).

The enzymes involved in DG formation by the 150,000 g supernatant from rapeseed were soluble but since their substrates and products are lipidic it was of interest to study the effect of detergents on the process. In Figure 4, the effect of various concentrations of three commonly used detergents, Triton X-100, sodium cholate and n-octyl β-D glucopyranoside, on DG formation from [^{14}C]oleoyl-CoA are shown. Triton X-100 inhibited DG synthesis by 90% even at relatively low concentrations while only inhibiting [^{14}C]oleoyl-CoA incorporation into total lipid by 50% with most of the balance of the [^{14}C] label accumulating in LPA. Therefore, Triton X-100 selectively inhibited DG formation while still allowing the synthesis of LPA to proceed. Although substantial inhibition of DG formation already was seen at Triton X-100 concentrations as low as 0.020%, it must be remembered that the CMC of this detergent is only 0.019%. Similar results were found with [^{14}C]G3P incorporation into DG in the presence of Triton X-100. Sodium cholate did not begin to inhibit DG formation until its concentration was almost 0.3% but since the CMC of cholate is very high (0.6%) in contrast to the other two detergents (34), this was not surprising. Unlike Triton X-100, sodium cholate did not cause any alteration in the labeling pattern of the lipid products of [^{14}C]oleoyl-CoA incubations, although the total incorporation of [^{14}C] label was reduced at higher detergent concentrations. In all of the cholate incubations, DG accounted for 70–80% of the total [^{14}C] label originally added as [^{14}C]oleoyl-CoA. Unlike the other two detergents, n-octyl β-D glucopyranoside did not inhibit either DG formation or total [^{14}C]oleoyl-CoA incorporation into lipid even at concentrations appreciably in excess of its CMC of 0.07% (24 mM). Interestingly, these results parallel the effects of sodium cholate and n-octyl β-D glucopyranoside on lysophospholipid acyltransferase activity in rapeseed microsomes (Murphy, D.J., unpublished data). The microsomal acyltransferases were relatively unaffected by n-octyl β-D glucopyranoside but substantially were inhibited by 100 μM sodium cholate.

These results show that developing embryos of rapeseed contain highly active enzymes capable of DG synthesis from oleoyl-CoA. The identity of the endogenous acyl acceptor is not yet clear since there was considerable DG synthesis from oleoyl-CoA even in the absence of exogenous acyl acceptors (Fig. 4). It is possible that a pool

of cytoplasmic G3P could be the endogenous acyl acceptor, in which case the lack of accumulation of LPA or PA intermediates would imply high levels of LPA-acyltransferase and PA phosphatase activities. The existence of soluble enzymes that utilize lipidic substrates is well-documented. For example, a soluble G3P acyltransferase recently has been purified from cocoa seeds (10), and soluble diacylglycerol kinases have been found in rat brain (35,36), bovine brain (37) and other tissues (38). In developing rapeseed, it was found that the oil-body, microsomal and soluble fractions each had sufficient diacylglycerol-forming activity to account for the rate in vivo. It is unwise to assign the subcellular localization of a metabolic pathway simply on the basis of in vitro assays of cell-free tissue fractions but the data presented here do raise the possibility that some reactions leading to TG synthesis may occur in the cytosol. The mechanism of oil-body formation still is controversial (17,23). It has been suggested that the endoplasmic reticulum is the site of TG synthesis (14,17-19). The deposition of TG in the lipid bilayer eventually will cause it to distend and bud off to form an oil-body surrounded by a proteinaceous monolayer of polar lipid (19,39,40). The results of this study are not entirely at variance with this mechanism since it is possible that the initial steps of TG synthesis could be catalyzed partly on soluble enzymes with only the final acylation of DG being confined exclusively to the endoplasmic reticulum.

Certain reservations about the above scheme, however, must be expressed. Oil bodies from a variety of seeds have a completely different polypeptide complement to that of the endoplasmic reticulum (Hills, M.J., and Murphy, D.J., personal communication, 22,41,42). Also, there is ultrastructural evidence for the initial deposition of oil in the ground cytoplasm of the cell near the surface of the plastids (22,43). At this stage, the oil droplets are not surrounded by a membrane, and they do not appear to be associated with the endoplasmic reticulum. Upon reaching a diameter of about 1 μm, the oil droplets become coated with a jacket of protein that apparently is derived from the endoplasmic reticulum. This protein jacket may or may not include a monolayer of polar lipid (22). Such a mechanism is consistent with the results of this present study. Oleate is produced in plastids and then is esterified to CoA to form oleoyl-CoA on the inner plastid envelope, resulting in the export of oleoyl-CoA into cytoplasm (44). Soluble acyltransferases in the cytoplasm will mediate the acylation of G3P to LPA and PA followed by phosphate removal by a soluble PA phosphatase to form DG. At this stage, the product will be a water-immiscible oil. The final acylation of DG to TG may be mediated by an enzyme made on the endoplasmic reticulum and transferred to the surface of the growing oil droplet.

It is possible that some triacylglycerol synthesis may occur on the endoplasmic reticulum. Indeed, it recently has been reported that a microsomal preparation from safflower seeds was able to form small, naked (without a protein coat) oil-bodies from acyl-CoA and G3P in vitro (45) although the subcellular origin of this membrane fraction was not studied. These investigations also have reported that microsomal fractions from developing rapesed were capable of TG formation at rates comparable with other oilseed species (Stymne, S. and Stobart, A.K., personal communication). In this study, it was found that oil-body, microsomal and soluble fractions of developing rapeseed embryos each had enough diacylglycerol-forming activity to account for the rate in vivo but that the soluble activity substantially was higher than that of the other two fractions. Although diacylglycerol synthesis is not confined to the soluble fraction, the possibility that some or even most of this synthesis occurs on soluble enzymes ought to be taken into account when consdering the mechanism of oil-body formation in oilseeds.

ACKNOWLEDGMENTS

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REFERENCES

1. Kennedy, E.P. (1961) *Fed. Proc. Am. Soc. Exp. Biol. 20,* 934-940.
2. Bertrams, M., and Heinz, E. (1976) *Planta 132,* 161-168.
3. Bertrams, M., and Heinz, E. (1981) *Plant Physiol. 68,* 653-657.
4. Frentzen, M., Heinz, E., McKeown, T., and Stumpf, P.O. (1983) *Eur. J. Biochem. 139,* 629-636.
5. Frentzen, M., Hares, W., and Schiburr, A. (1984) in *Structure, Function and Metabolism of Plant Lipids* (Siegentheler, P.A., and Eichenberger, E., eds.) pp. 105-110, Elsevier, Amsterdam.
6. Joyard, J., and Douce, R. (1977) *Biochim. Biophys. Acta 485,* 273-285.
7. Andrews, J., Ohlrogge, J.B., and Keegstra, K. (1985) *Plant Physiol. 78,* 459-465.
8. Martin, B.A., and Wilson, R.F. (1983) *Lipids 18,* 1-6.
9. Martin, B.A., and Wilson, R.F. (1984) *Lipids 19,* 117-121.
10. Fritz, P.J., Kauffman, J.M., Robertson, C.A., and Wilson, M.R. (1986) *J. Biol. Chem. 261,* 194-199.
11. Ichihara, K. (1984) *Arch. Biochem. Biophys. 232,* 685-698.
12. Ichihara, K., and Noda, M. (1981) *Phytochemistry 20,* 1245-1249.
13. Ichihara, K., and Noda, M. (1982) *Phytochemistry 21,* 1895-1901.
14. Cao, Y., and Huang, A.H.C. (1986) *Plant Physiol. 82,* 813-820.
15. Gurr, M.I., Blades, J., Appleby, R.S., Smith, C.G., Robinson, M.P., and Nichols, B.W. (1974) *Eur. J. Biochem. 43,* 281-290.
16. Murphy, D.J., and Mukherjee, K.D. (1987) *Lipids 22,* 293-298.
17. Frey-Wyssling, A., Grieshaber, E., and Mühletheler, K. (1963) *J. Ultrastr. Res. 8,* 506-516.
18. Schwarzenbach, A.M. (1971) *Cytobiologie 4,* 145-147.
19. Wanner, G., and Theimer, R.R. (1978) *Planta 140,* 163-169.
20. Rest, J.A., and Vaughan, J.G. (1972) *Planta 105,* 245-262.
21. Smith, C.G. (1974) *Planta 119,* 125-142.
22. Bergfeld, R., Hong, Y.N., Kuhnl, T., and Schopfer, P. (1978) *Planta 143,* 297-307.
23. Gurr, M.I. (1980) in *Biochemistry of Plants* (Stumpf, P.K., and Conn, E.E., eds.) Vol. 4, pp. 205-248, Academic Press, New York, NY.
24. Ichihara, K. (1982) *Agric. Biol. Chem. 46,* 1767-1773.
25. Ohlrogge, J.B., Kuhn, D.N., and Stumpf, P.K. (1979) *Proc. Natl. Acad. Sci. USA 76,* 1194-1198.
26. Murphy, D.J., Mukherjee, K.D., and Latzko, E.T. (1984) *Planta 161,* 249-254.
27. Hauser, G., and Eichberg, J. (1975) *J. Biol. Chem. 250,* 105-112.
28. Markwell, M.A.K., Haas, S.M., Tolbert, N.E., and Bieber, L.L. (1981) *Methods Enzymol. 72,* 296-303.
29. Mukherjee, K.D. (1986) *Planta 157,* 279-283.
30. Joyard, J., and Douce, R. (1979) *FEBS Lett. 102,* 147-150.
31. Slack, C.R., Campbell, L.C., Browse, J.A., and Roughan, P.G. (1983) *Biochim. Biophys. Acta 754,* 10-20.
32. Slack, C.R., Roughan, P.G., Browse, J.A., and Gardiner, S.E. (1985) *Biochim. Biophys. Acta 833,* 438-448.
33. Moreau, R.A., and Stumpf, P.K. (1982) *Plant Physiol. 69,* 1293-1297.
34. Helenius, A., and Simons, K. (1975) *Biochim. Biophys. Acta 415,* 29-79.

35. Lapetina, E.G., and Hawthorne, J.N. (1971) *Biochem. J. 122*, 171-177.
36. Bishop, H.H., and Strickland, K.P. (1980) *Lipids 15*, 285-291.
37. Lin, C-H., Bishop, H., and Strickland, K.P. (1986) *Lipids 21*, 206-211.
38. Kanoh, H., Kondoh, H., and Ono, T. (1983) *J. Biol. Chem. 258*, 1767-1774.
39. Wanner, G., Formanek, H., and Theimer, R.R. (1981) *Planta 140*, 163-169.
40. Gurr, M.I. (1980) in *The Biochemistry of Plants* (Stumpf, P.K., and Conn, E.E., eds.) Vol. 4, pp. 205-248, Academic Press, New York, NY.
41. Slack, C.R., Bertaud, W.S., Shaw, B.D., Holland, R., Browse, J., and Wright, H. (1980) *Biochem. J. 190*, 551-561.
42. Moreau, R.A., Liu, K.D.F., and Huang, A.H.C. (1980) *Plant Physiol. 65*, 1176-1180.
43. Ichihara, K. (1982) *Agric. Biol. Chem. 46*, 1767-1773.
44. Douce, R., and Joyard, J. (1980) in *The Biochemistry of Plants* (Stumpf, P.K., Conn, E.E., eds.) Vol. 4, pp. 321-362, Academic Press, New York, NY.
45. Stobart, A.K., Stymne, S. and Hoglund, S. (1986) *Planta 169*, 33-37.

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Effects of Dietary Fiber in Vervet Monkeys Fed "Western" Diets

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Male Vervet monkeys (7/treatment) were fed a "Western" diet containing 46.2% calories as fat, 39.8% as carbohydrate and 14.0% as protein. The diet was augmented with 10% cellulose or 10% pectin. A third (control) group of seven monkeys was fed a commercial ration augmented with fruit and bread. After 34 weeks, serum cholesterol levels were elevated significantly in the two test groups compared with the controls but there was no difference between the two fiber-fed groups. Serum triglycerides were unaffected. Liver cholesterol levels were the same in all three groups but liver triglyceride levels were lower in the monkeys fed cellulose. Biliary lipids were similar in all three groups as were the calculated lithogenic indices. The average aortic sudanophilia (percent of total area) in the three groups was cellulose, 10.6 ± 2.5; pectin, 8.1 ± 2.5; and control, 1.1 ± 0.4. One animal in each of the groups fed "Western" diet exhibited an atherosclerotic plaque. The results indicate that there is no difference between pectin and cellulose with regard to their effects on either lipidemia or aortic sudanophilia in Vervet monkeys fed a Western-type diet.
***Lipids* 23, 164–168 (1988).**

A diet similar to that usually eaten in industrially developed countries (often called a Western diet) has been shown to lead to aortic sudanophilia and atherosclerosis when fed to Rhesus (1) or Vervet (2) monkeys. The "Western" diet usually is compared with a "prudent" diet, which is less sudanophilic. The effects of different dietary fibers on lipidemia and sudanophilia have been compared in monkeys fed semipurified diets (3–5) but not under conditions in which a diet of the type eaten in developed countries has been used. This communication describes a comparison of the effects of cellulose and pectin on lipidemia and aortic sudanophilia in Vervet monkeys fed a "Western" diet.

MATERIALS AND METHODS

Male Vervet monkeys *(Cercopithecus aethiops)* were used throughout. The monkeys (average starting weight 5.65 kg) were maintained in individual cages (one animal per cage) and daily were fed 150 gm of diet. They had ad libitum access to water. There were three dietary groups of seven monkeys each. The diet is described in Table 1. The diet provided 245 Kcal/100 gm; its caloric composition was 46.2% as fat, 39.8% as carbohydrate and 14.0% as protein. The diet was adjusted to provide 150 mg of cholesterol per 100 gm or 0.61 mg of cholesterol per Kcal. The ratios of animal to vegetable protein and fat were 6.39 and 5.31, respectively. The ratio of starch to sugar was 1.63. The fibers (cellulose or apple pectin) were added at a level of 10%. A third group was fed a commercial ration. The commercial diet was obtained from Epol (Pty) Ltd. (Johannesburg, South Africa) and contained 20% protein, 5% fat and about 65% carbohydrate. The cellulose and pectin used also were obtained from Epol (Pty) Ltd. The dietary composition was calculated from the tables of Paul and Southgate (6) and confirmed by analysis. The fatty acid composition of the two diets ("Western" and control) are presented in Table 2.

The monkeys were bled at the beginning of the study and then were bled at monthly intervals until the termination of the study 34 weeks later. Serum was analyzed for total cholesterol (7), triglycerides (8) and phospholipids (9). The high density lipoprotein (HDL) cholesterol was

TABLE 1

"Western" Diet*

Ingredient	Grams/100 gm
Ground beef	16.2
Eggs	8.1
Butter	3.3
Peanut oil	1.6
Potato	12.5
Carrot	12.5
Banana	6.3
Bread	8.0
Sugar	9.6
Skim milk powder	11.9
Fiber	10.0

*The ingredients were mixed thoroughly and baked at 160 C for two hr. Cholesterol (36 mg) was added per 100 g diet to provide 150 mg cholesterol/100 g. The diet provided 245 Kcal/100 g with 46.2% of calories as fat, 39.8% as carbohydrate and 14.0% as protein. Cholesterol content: 150 mg/100 g or 0.61 mg/Kcal cholesterol added to give a closer approximation to the cholesterol content of diets eaten by developed populations. The fiber was cellulose or apple pectin.

TABLE 2

Fatty Acids in "Western" and Control Diets

	% Recovered	
Fatty acid	Western diet	Control
12:0	0.65	0.09
14:0	4.28	1.96
16:0	22.98	18.60
16:1	3.45	3.18
18:0	12.22	11.54
18:1	37.80	36.34
18:2	10.93	20.37
Other	7.69	7.12

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Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

analyzed after precipitation of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) by sodium phosphotungstate (10). At the termination of the experiment, the monkeys were killed by exsanguination while under deep anesthesia. Livers were excised and weighed, and aliquots were extracted with chloroform/methanol 2:1 (11). The extracted lipids were separated by thin layer chromatography on silic acid. The various fractions were extracted from the silica gel (12) and analyzed colorimetrically. Biliary bile was aspirated and deproteinized with ethanol. The levels of cholesterol (13), phospholipid (9) and total bile acids (Sterognost 3αPHO enzyme kit) present in the bile were determined and the lithogenic index calculated (14). The aortas were fixed, stained with Sudan IV and the extent of lipid deposition assessed. The histological procedures have been described (4,15). Data were analyzed by Student's t-test (16).

RESULTS

The serum lipid levels averaged over the duration of the study are detailed in Table 3. We and others have reported on temporal variations in serum lipid levels in primates (17–20). Thus, we have opted to present the average levels over the course of the study and the levels determined at the end of the experiment (Table 4). Table 3 shows that total serum cholesterol levels in monkeys fed the Western diet were elevated significantly as compared with the starting or the final control values ($p < 0.001$). The LDL cholesterol levels also were elevated significantly (<0.001), and the LDL cholesterol levels in the monkeys fed pectin were significantly ($p < 0.05$) lower than in those fed cellulose. The HDL/LDL cholesterol ratio was reduced significantly in monkeys fed the Western diet. Triglyceride levels were unaffected by the dietary regimens.

The necropsy data are summarized in Table 4. Weight gains and liver weights were similar in all three groups. Serum cholesterol and phospholipid levels were elevated significantly in the two groups fed the Western diet. We did not assay LDL or HDL cholesterol levels in the sera obtained at autopsy but the HDL/LDL cholesterol ratios in sera obtained three weeks earlier were cellulose, 0.20 ± 0.05; pectin, 0.28 ± 0.03; and control, 0.52 ± 0.09. The ratio in the control group was significantly higher than

TABLE 3

Average Serum Lipids (mg/dl ± SEM) of Monkeys Fed "Western" Diet Plus 10% Cellulose or Apple Pectin for 34 Weeks

	Group		
	Cellulose	Pectin	Control
Cholesterol (113 ± 3)*	300 ± 30[a,b]	223 ± 16[a,c]	113 ± 9[b,c]
LDL Cholesterol (59 ± 2)	239 ± 33[a,b]	155 ± 17[a,c]	63 ± 8[b,c]
HDL Cholesterol (43 ± 1)	50 ± 6	58 ± 2[a]	39 ± 2[a]
HDL/LDL (0.79 ± 0.04)	0.31 ± 0.04[a]	0.43 ± 0.05[b]	0.74 ± 0.07[a,b]
Triglycerides (54 ± 3)	51 ± 4	50 ± 2	46 ± 5

*Average starting value for entire group.

Values in horizontal row bearing same letters are significantly ($p \leq 0.05$) different.

TABLE 4

Necropsy Data: Vervet Monkeys Fed "Western" Diet Plus 10% Cellulose or Pectin for 34 Weeks (data ± SEM)

	Group		
	Cellulose	Pectin	Control
Weight gain (Kg)	0.4 ± 0.2	0.3 ± 0.2	0.6 ± 0.3
Liver weight (g)	116 ± 2	109 ± 8	111 ± 6
Relative liver weight	1.88 ± 0.06	1.78 ± 0.10	1.97 ± 0.08
Serum lipids (mg/dl)			
Cholesterol	163 ± 12[a]	185 ± 11[b]	119 ± 10[a,b]
Triglyceride	22 ± 2	27 ± 3	21 ± 1
Phospholipid	269 ± 14[a]	314 ± 27[b]	205 ± 11[a,b]
Liver lipids (g/100 g)			
Cholesterol	0.42 ± 0.03	0.46 ± 0.03	0.40 ± 0.04
% Ester	13 ± 2	20 ± 5	10 ± 2
Triglycerides	0.45 ± 0.08	1.26 ± 0.44	1.08 ± 0.40
Phospholipids	8.93 ± 0.63	8.96 ± 0.23	8.69 ± 0.72
Biliary bile (mg/ml)			
Cholesterol	2.60 ± 0.25	2.95 ± 0.24	2.39 ± 0.32
Phospholipid	20.1 ± 1.57	22.6 ± 1.34	19.1 ± 2.23
Bile acid	97.6 ± 8.70[a]	126.3 ± 2.92[a]	117.9 ± 5.32
Lithogenic index	0.058 ± 0.008	0.049 ± 0.005	0.046 ± 0.004

Values in horizontal row bearing same letters are significantly ($p \leq 0.05$) different.

that in either of the other groups. Terminal triglyceride levels were unexplicably low in all three groups. Liver total cholesterol levels were similar in all three groups but levels of esterified cholesterol were elevated slightly in livers of the monkeys fed the pectin-containing diet. Liver triglyceride levels were 60% lower in the livers of the monkeys fed cellulose than in the other two groups but the differences were not significant. The lithogenic index, calculated from assayed levels of biliary cholesterol, phospholipid and total bile acid, was low in all three groups. Biliary cholesterol and phospholipids were similar in all three groups but the bile acid content of the cellulose-fed group was 23% lower than that in the pectin-fed group ($p < 0.01$) and 17% lower than that in the control group (not significant).

Fecal weight was monitored over the last six days of the study. Wet and dry weights of the feces were similar in all three groups ranging from 42–45 g/day and 7.5–8.5 g/day, respectively. Water content of the feces was 82.1 ± 0.43%.

The outlines of sudanophilic areas are shown in Figures 1–3. The area was determined independently by three investigators, and the average values for each aorta are given in Table 5. Average aortic sudanophilia in monkeys fed the Western diet plus cellulose was 31% (not significant) higher than in those fed pectin. Monkeys fed the control diet exhibited very little sudanophilia, 90 and 86% less than was seen in the cellulose or pectin groups, respectively. One animal in each of the groups fed Western diet exhibited an atherosclerotic plaque.

DISCUSSION

In general, pectin has been found to be hypocholesterolemic in rats (21–23), rabbits (24), chickens (25) and guinea pigs (21). It also is hypocholesterolemic in man (26,27). Cellulose, on the other hand, has no hypocholesterolemic effect and often is hypercholesterolemic in rats (28–30), rabbits (31), chickens (32) and guinea pigs (33).

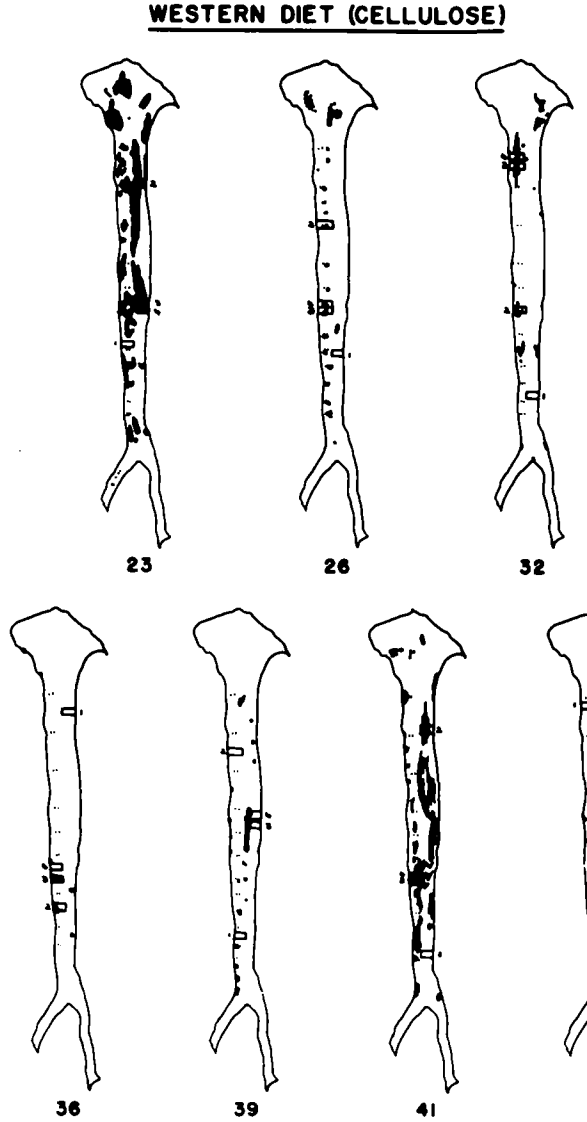

FIG. 1. Schematic representation of aortic sudanophilia in Vervet monkeys fed a "Western" diet plus 10% cellulose.

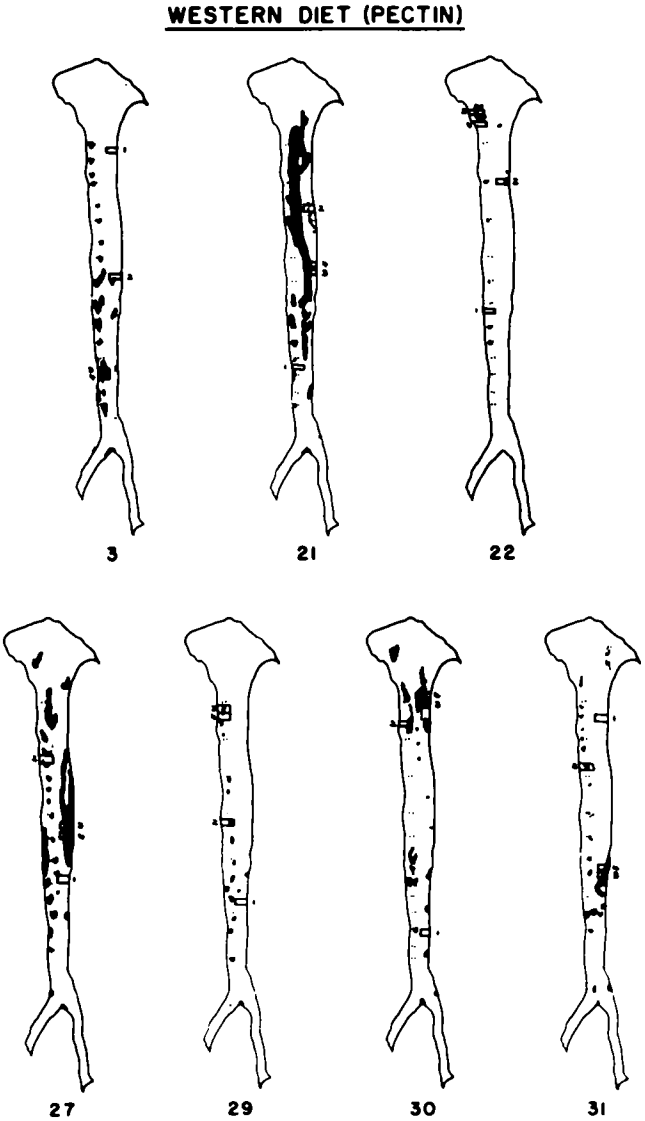

FIG. 2. Schematic representation of aortic sudanophilia in Vervet monkeys fed a "Western" diet plus 10% pectin.

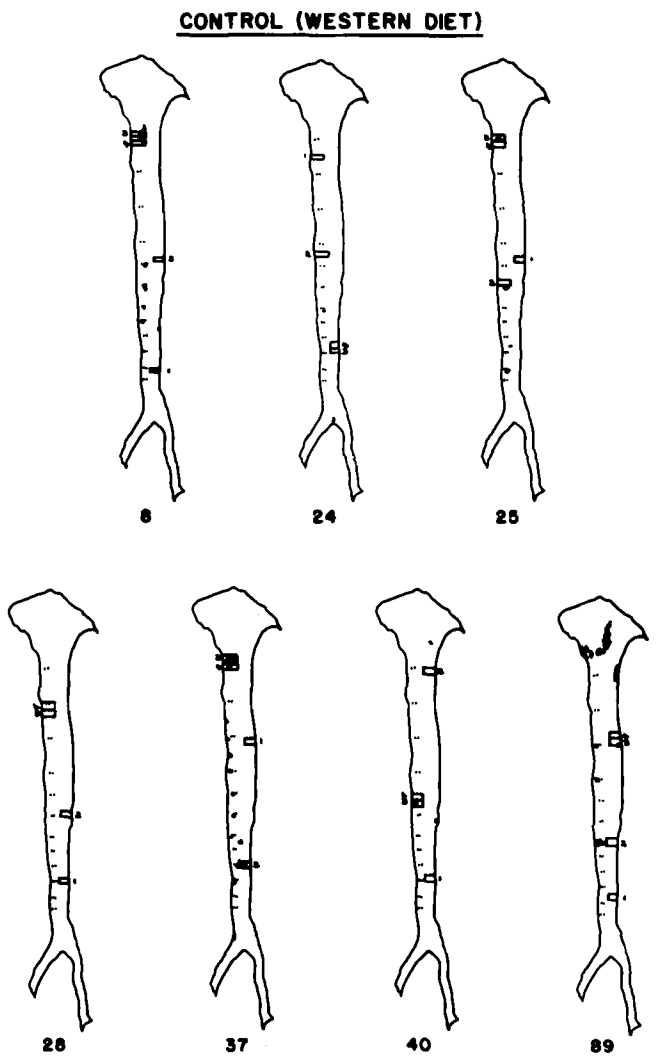

FIG. 3. Schematic representation of aortic sudanophilia in Vervet monkeys fed commercial ration.

TABLE 5

Average Aortic Sudanophilia (± SEM) in Vervet Monkeys Fed "Western" Diets Containing 10% Cellulose or Apple Pectin

Cellulose		Pectin		Control	
Monkey no.	%*	Monkey no.	%*	Monkey no.	%*
23	34	3	8[a]	8	1
26	5	21	20	24	0
32	7	22	2	25	1
36	2	27	14	28	0
39	3	29	2	37	1
41	21[a]	30	7	40	2
44	2	31	4	89	3
AVG: 10.6 ± 4.6		8.1 ± 2.5		1.1 ± 0.4	

*Average of evaluation by three independent investigators.
[a]Monkey bearing atherosclerotic plaque.

Cellulose has little influence on cholesterol levels in man (34). Compared to cellulose, pectin is antiatherogenic in chickens (35), and rabbits fed cellulose exhibit higher cholesterol levels and more severe atherosclerosis than those fed alfalfa (36). Pectin has been reported to raise cholesterol levels in pigs (37).

There are few reports of the effects of fiber on lipidemia or atherosclerosis in primates. We (20) found that baboons fed a semipurified diet in which the fiber was cellulose exhibited higher serum lipid levels and more severe aortic sudanophilia than those fed a diet consisting of commercial ration, bread and fruit. Vervet monkeys fed a semipurified diet containing no cholesterol exhibited higher serum levels than monkeys fed similar diets in which the fiber was alfalfa or wheat straw (4). Recently (5), we fed Vervet monkeys semipurified diets with or without 0.1% cholesterol and either cellulose or pectin, both fed as 15% of the diet. There were no significant differences in serum cholesterol levels or aortic sudanophilia, although that observed in the pectin-fed animals was higher. In this study we found that a Western diet is cholesterolemic and sudanophilic for Vervet monkeys. The type of fiber present in the diet (comparing only cellulose and pectin) does not affect weight gain. Comparison of the two fibers shows no effect on serum lipids or liver cholesterol but liver triglycerides are lower in monkeys fed cellulose. Severity of aortic sudanophilia was similar in the two fiber-fed groups, although the average was lower in aortas of monkeys fed pectin. One monkey in each group exhibited an atherosclerotic plaque. The results indicate that under the conditions of this experiment, cellulose and pectin have equivalent effects. Fiber-rich diets have been shown to enhance excretion of endogenously synthesized or dietary cholesterol in rabbits (38), and pectin has been shown to enhance bile acid excretion in rats (22) and man (26). This study is the second to show that dietary pectin does not influence lipidemia in Vervet monkeys. More work is needed to identify the mechanism(s) by which pectin fails to affect lipidemia in Vervet monkeys and to ascertain whether this lack of effect is restricted to the Vervet monkeys or is general for subhuman primates.

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REFERENCES

1. Wissler, R.W., Vesselinovitch, D., Hughes, R., Turner, D., and Frazier, L. (1983) *Exp. Molec. Pathol. 38*, 117-136.
2. Fincham, J.E., Woodruff, C.W., van Wyk, M.J., Capatos, D., Weight, M.J., Kritchevsky, D., and Rossouw, J.E. *Atherosclerosis 66*, 205-215.
3. Malinow, M.R., McLaughlin, P., Papworth, L., Naito, H.K., and Lewis, L.A. (1976) *Am. J. Clin. Nutr. 29*, 905-911.
4. Kritchevsky, D., Davidson, L.M., Krendel, D.A., Van der Watt, J.J., Russell, D., Friedland, S., and Mendelsohn, D. (1981) *Ann. Nutr. Metabol. 25*, 125-136.
5. Kritchevsky, D., Davidson, L.M., Goodman, G.T., Tepper, S.A., and Mendelsohn, D. (1986) *Lipids 21*, 338-341.
6. Paul, A.A., and Southgate, D.A.T. (1978) *The Composition of Foods*, Elsevier/North Holland Medical Press, Amsterdam.
7. Zlatkis, A., and Zak, B. (1969) *Anal. Biochem. 29*, 143-148.
8. Van Handel, E., and Zilversmit, D.B. (1957) *J. Lab. Clin. Med. 50*, 152-157.

9. Sokoloff, L., and Rothblat, G.H. (1974) *Proc. Soc. Exp. Biol. Med. 146,* 1166-1172.
10. Burstein, M., Scholnick, H.R., and Morfin, R. (1970) *J. Lipid Res. 11,* 583-595.
11. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
12. Kritchevsky, D., and Malhotra, S. (1970) *J. Chromatog. 52,* 498-499.
13. Zak, B., Dickenman, R.C., White, E.G., Burnett, H., and Cherney, P.J. (1954) *Am. J. Clin. Pathol. 24,* 1307-1315.
14. Thomas, P.J., and Hofmann, A.F. (1973) *Gastroenterology 65,* 698-700.
15. Kritchevsky, D., Davidson, L.M., Kim, H.K., Krendel, D.A., Malhotra, S.M., Van der Watt, J.J., du Plessis, J.P., Winter, P.A.D., Ipp, T., Mendelsohn, D., and Bersohn, I. (1977) *Exp. Molec. Pathol. 26,* 28-51.
16. Sokal, R.R., and Rohlf, F.J. (1969) *Introduction to Biostatistics,* pp. 170-175, W.H. Freeman and Co., San Francisco, CA.
17. Kritchevsky, D., and McCandless, R.F.J. (1957) *Proc. Soc. Exp. Biol. Med. 95,* 152-154.
18. Strong, J.P., Radelat, P.B., Guidry, M.A., and McMahan, C.A. (1964) *Circulation Res. 14,* 367-372.
19. Kritchevsky, D., Kritchevsky, E.S., Nair, P.P., Jastremsky, J.A., and Shapiro, I.L. (1967) *Nutr. Dieta 9,* 283-299.
20. Kritchevsky, D., Davidson, L.M., Shapiro, I.L., Kim, H.K., Kitagawa, M., Malhotra, S., Nair, P.P., Clarkson, T.B., Bersohn, I., and Winter, P.A.D. (1974) *Am. J. Clin. Nutr. 27,* 29-50.
21. Wells, A.F., and Ershoff, B.H. (1962) *Proc. Soc. Exp. Biol. Med. 111,* 147-149.
22. Leveille, G.A., and Sauberlich, H.E. (1966) *J. Nutr. 88,* 209-214.
23. Riccardi, B.A., and Fahrenbach, M.J. (1967) *Proc. Soc. Exp. Biol. Med. 124,* 749-752.
24. Berenson, L.M., Bhanduru, R.R., Radhakrishnamurty, B., Srinivasan, S.R., and Berenson, G.S. (1975) *Life Sci. 16,* 1533-1544.
25. Fahrenbach, M.J., Riccardi, B.A., and Grant, W.C. (1966) *Proc. Soc. Exp. Biol. Med. 123,* 321-326.
26. Kay, R.M., and Truswell, A.S. (1977) *Am. J. Clin. Nutr. 30,* 171-175.
27. Miettinen, T.A., and Tarpila, S. (1977) *Clin. Chim. Acta 79,* 471-477.
28. Kritchevsky, D., Tepper, S.A., and Story, J.A. (1974) *Nutr. Rep. Int. 9,* 301-308.
29. Tsai, A.C., Elias, J., Keeley, J.J., Lin, R.S.C., and Robson, J.R.K. (1976) *J. Nutr. 106,* 118-123.
30. Story, J.A., Baldino, A., Czarnecki, S.K., and Kritchevsky, D. (1981) *Nutr. Rep. Int. 24,* 1213-1219.
31. Hamilton, R.M.G., and Carroll, K.K. (1976) *Atherosclerosis 24,* 47-62.
32. Menge, H., Littlefield, L.H., Frobish, L.T., and Weinland, B.T. (1974) *J. Nutr. 104,* 1554-1566.
33. Fahey, G.C. Jr., Muller, B.L., and Hadfield, H.W. (1979) *J. Nutr. 109,* 77-83.
34. Behall, K.M., Lee, K.H., and Moser, P.B. (1984) *Am. J. Clin. Nutr. 39,* 204-214.
35. Fisher, H., Soller, W.G., and Grimminger, P. (1966) *J. Atheroscler. Res. 6,* 292-298.
36. Kritchevsky, D., Tepper, S.A., Williams, D.E., and Story, J.A. (1977) *Atherosclerosis 26,* 397-403.
37. Fausch, H.D., and Anderson, T.A. (1965) *J. Nutr. 85,* 145-149.
38. Kritchevsky, D., Tepper, S.A., Kim, H.K., Moses, D.E., and Story, J.A. (1975) *Exp. Molec. Pathol. 22,* 11-19.

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Effects of Cholesterol Feeding to Maternal Rats on Metabolism of Cholesterol and Bile Acids in the Dams and Their Offspring

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The influence of feeding cholesterol to rats during pregnancy and postpartum (from the 11th day of gestation to the third day after delivery) on the serum and hepatic cholesterol levels and on the bile acid composition in the pool and in the liver in relationship to the dams and their pups was examined. The hepatic content of cholesterol in both dam and offspring increased during cholesterol feeding without any changes in serum cholesterol level. In the dams, mainly the esterified cholesterol was increased; in the pups, mainly the free cholesterol was increased. Cholesterol feeding led to a pronounced increase in the pool of β-muricholic acid and a relative decrease in the lithocholic acid concentration in pregnant rats. In fetal rats, the chenodeoxycholic acid pool was increased by cholesterol intake. The lithocholic acid pool was larger in the postpartum rats fed cholesterol than in the controls, while the concentration of α- and β-muricholic acids was decreased. The neonates of cholesterol-fed dams had a larger pool of chenodeoxycholic acid but a smaller pool of β-muricholic acid. These results suggest that the metabolism of cholesterol and of bile acids in dams and their offspring respond differently to cholesterol intake. *Lipids 23,* 169–177 (1988).

In adult rats, cholesterol feeding caused a marked accumulation of cholesterol in liver and an increase in chenodeoxycholic, α-muricholic and β-muricholic acids in the bile acid pool with a relative decrease in cholic acid (1–3).

Such a metabolic alteration in the pregnant and the postpartum rats is likely to affect the metabolism of cholesterol and bile acids in the developing fetuses and in early neonatal life because cholesterol can pass through the placenta from the dam to the fetuses and also can be supplied to the neonates via milk through the mammary glands of the dam (4–7). In addition, a significant fraction of the bile acid pool in the fetuses might be derived from the dam (8,9).

We have carried out a series of experiments to study the effect of cholesterol feeding to the dam on the maternal metabolism of cholesterol and bile acids and to investigate whether these changes, if any, could affect the metabolism of cholesterol and bile acids in the fetal and the neonatal rats.

MATERIALS AND METHODS

Materials. β-Muricholic acid and ω-muricholic acid were synthesized according to the literature (10). Cholesterol (99.5% pure) was obtained from Wako Pure Chemical Industries (Osaka). Sodium tauro[24-^{14}C]cholate (44.5 mCi/mmol) was purchased from the New England Nuclear Corp. (Boston, MA). Reagents and organic solvents were of analytical grade, the latter being distilled once before use, if necessary.

Animals and diet. Female Wistar strain rats on the 11th day of gestation, each weighing 230–280 g, were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka), and assigned to one of three groups. One group was fed a commercial rat diet (Clea CA-1, Nihon Clea Co., Tokyo); the second group was fed the same diet supplemented with 0.5% cholesterol; and the third group was fed a 2.5% cholesterol-supplemented diet. The diets contained 25.6% protein, 4.6% fat, 4.0% fiber and all vitamins at recommended levels. The cholesterol-supplemented diets also were prepared by the same manufacturer. The stock diet contained 1.36 ± 0.15 mg (mean ± SD, n = 4) of cholesterol and 137 ± 56 μg (mean ± SD, n = 4) of cholic acid/g, analyzed in our laboratory. The animals were maintained on the respective diets ad libitum during a period from the 11th day of pregnancy to the third postpartum day. They were housed in individual cages, and kept in an air-conditioned room (23 C) lighted from 6 a.m. to 6 p.m.

Twenty-day pregnant rats were killed under ethereal anesthesia, and 20-day prenatal rats were removed from the uterus. Three-day postpartum rats and three-day neonatal rats also were killed in the same way. Blood samples were obtained from maternal, fetal and neonatal rats by cardiac puncture, and the serum was separated from the blood. The liver and the intestine were excised separately and weighed. Samples from the fetal and the neonatal rats were pooled for analysis (three to five samples per pool). All operations for sampling were performed between 9 and 11 a.m., and all samples were stored at −20 C until analysis.

Analysis of cholesterol. Serum total and free cholesterol were determined enzymatically with Determiner TC'5' and FC (Kyowa Medex Co., Tokyo), respectively. A portion of the liver from maternal rats was extracted with chloroform/methanol (2:1, v/v) (11). The extracted lipids were chromatographed on silicic acid (AR, 100 mesh, Mallinckrodt Chemical Works, St. Louis, MO); the cholesterol fraction was eluted with hexane/chloroform (3:2, v/v). The cholesterol fraction was further separated into esterified and free cholesterol (12). Esterified cholesterol was saponified at 60 C for 30 min with 2 N KOH/propanol and extracted with hexane. Cholesterol was determined by gas chromatography as described below.

Fetal and neonatal liver were homogenized with ether/ethanol (1:1, v/v) and left to stand overnight. The tauro[24-^{14}C]cholate was added to some samples before homogenization as a recovery standard for bile acids. The crude lipid extract was partitioned between ether/heptane (1:1, v/v) and 50% aqueous ethanol (13). The ether/heptane extract was analyzed for esterified and free cholesterol as described above. The residual matter left after extraction with ether/ethanol and the aqueous ethanol extract

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in the partitioning system were used for analysis of bile acids.

Analysis of bile acids. Another portion of the liver from the dams was homogenized in physiological saline and extracted by refluxing in 95% ethanol containing 0.5% ammonium hydroxide (14). The residue of fetal and neonatal liver remaining after the ether/ethanol extraction was extracted similarly, and the extract was combined with the residue of the aqueous ethanol layer in the partition system for the crude lipid extract from liver as mentioned above. The liver extract was dissolved in 0.1 N sodium hydroxide in physiological saline and chromatographed on Amberlite XAD-2 (15). Bile acids were eluted with 0.5% ammonium hydroxide in 95% ethanol, subjected to solvolysis in methanol/acetone (1:9, v/v) containing hydrochloric acid (16), and hydrolyzed in 2 N KOH solution at 130 C for three hr. Free bile acids were extracted with ether after acidification, methylated with diazomethane, and acetylated in acetic anhydride (17). In some samples of fetal and neonatal liver, the extraction efficiency of bile acids was checked by the recovery of [24-^{14}C]cholic acid added as its taurine-conjugate before extraction as mentioned above. Methyl ester acetates of bile acids were quantitated by gas chromatography. Intestinal tracts plus their contents were homogenized and refluxed in 20 volumes of 90% ethanol for two hr. Refluxing was repeated two times, and the pooled extract was analyzed as described above.

Gas chromatography. Samples dissolved in acetone were gas chromatographed in a Shimadzu Model GC-3BF unit with a coiled glass column (3 mm × 1.75 m) packed with 1.5% OV-1 on a Gas Chrom Q (100–120 mesh). Operating temperatures were injector, 255 C; column and detector, 230 C. Nitrogen was used as the carrier gas at a flow rate of 46.2 ml/min. Bile acids and free cholesterol were identified tentatively by comparing their retention times relative to deoxycholic acid (= 1.00) with those of the respective standards. Quantitative determination was made by comparing the peak area with those of a known amount of the corresponding standards using a Chromatopac C-R1A (Shimadzu Corp., Kyoto). Unidentified peaks suspected to be a bile acid were calculated as cholic acid.

Statistical method. Control and experimental values were compared using Student's t-test.

RESULTS

Metabolism of cholesterol and bile acids in the dam. Table 1 shows daily food intake, body weight, liver weight, and hepatic and serum levels of cholesterol for 20-day pregnant rats. The diet intake, body weight and liver weight in the cholesterol-fed groups did not differ from the corresponding value in the control group. There also were no significant differences in the level of any cholesterol fraction in serum between the control group and either cholesterol-fed group. The esterified cholesterol level in liver was higher in the cholesterol-fed groups than in the control group. In addition, the level of free cholesterol was also higher in the 2.5% cholesterol-fed group than in the control group.

Table 2 shows the composition of the bile acid pool (the sum of bile acids in the intestinal tract with its contents and those in the liver). The amount of chenodeoxycholic acid ($p < 0.10$), β-muricholic acid and others was greater in both cholesterol-fed groups than in the control group. Besides, in the 2.5% cholesterol-fed group, the amounts of lithocholic acid and hyodeoxycholic acid were less than those in the control group. In all groups, more than 95% of the total bile acid pool was distributed in the intestinal tract (data not shown).

Table 3 shows the hepatic content of bile acids. β-Muricholic acid and chenodeoxycholic acid ($p < 0.10$) were more abundant in the 2.5% cholesterol-fed group than in the control group. Also, in the 0.5% cholesterol-fed group, more β-muricholic acid ($p < 0.10$) was present as compared with the control.

TABLE 1

Diet Intake, Body Weight, Liver Weight and Cholesterol Levels in 20-Day Pregnant Rats

Observations	Rat group		
	Control	0.5% Cholesterol-fed	2.5% Cholesterol-fed
Diet intake (g/day)	24 ± 3[d] (4)[e]	27 ± 2 (4)	27 ± 1 (6)
Body weight (g)	364 ± 6 (4)	369 ± 14 (4)	370 ± 9 (6)
Liver weight[a] (g)	11.1 ± 0.3 (4)	16.0 ± 1.0 (4)	14.5 ± 0.4 (6)
Serum cholesterol (mg/dl)			
Free	24.8 ± 3.4 (4)	33.7 ± 3.3 (3)	29.4 ± 3.4 (5)
Ester[b]	89.2 ± 10.9	101.1 ± 3.8	114.6 ± 10.8
Total	114.0 ± 10.4 (4)	134.8 ± 1.8 (3)	144.0 ± 10.2 (5)
Hepatic cholesterol (mg/g liver[a])			
Free	0.74 ± 0.07 (4)	0.89 ± 0.03 (3)	1.44 ± 0.13[f] (4)
Ester	0.10 ± 0.00 (4)	0.46 ± 0.02[f] (3)	0.45 ± 0.07[f] (4)
Total[c]	0.84 ± 0.07	1.34 ± 0.03	1.89 ± 0.15

[a]Wet weight.

[b]The difference between total and free cholesterol.

[c]The sum of free and ester cholesterol.

[d]Means ± SEM.

[e]The number of samples studied.

[f]Significantly different from controls, $p < 0.05$.

TABLE 2

Composition of Bile Acid Pool in 20-Day Pregnant Rats

Bile acid	Pool size (mg/100 g body weight) Control (N = 4)	0.5% Cholesterol-fed (N = 5)	2.5% Cholesterol-fed (N = 4)
Lithocholic	0.35 ± 0.05[b]	0.41 ± 0.10	0.14 ± 0.02[c]
Deoxycholic	1.15 ± 0.23	1.61 ± 0.16	1.42 ± 0.16
Chenodeoxycholic	1.29 ± 0.15	2.00 ± 0.28	1.74 ± 0.13
Hyodeoxycholic	1.32 ± 0.16	1.72 ± 0.17	0.48 ± 0.07[c]
Cholic	7.20 ± 1.38	5.34 ± 0.79	4.27 ± 0.21
α-Muricholic	1.12 ± 0.17	1.52 ± 0.14	1.33 ± 0.10
β-Muricholic	0.10 ± 0.03	0.57 ± 0.08[c]	1.36 ± 0.17[c]
ω-Muricholic	0.26 ± 0.09	0.67 ± 0.12	0.44 ± 0.04
Others[a]	0.71 ± 0.08	2.02 ± 0.14[c]	1.08 ± 0.06[c]
Total	13.50 ± 1.43	15.85 ± 0.91	12.27 ± 0.37

[a]The sum of keto bile acids and minor unidentified components.
[b]Means ± SEM.
[c]Significantly different from controls, $p < 0.05$.

TABLE 3

Hepatic Bile Acids in 20-Day Pregnant Rats

Bile acid	Hepatic content (μg/g liver weight) Control (N = 4)	0.5% Cholesterol-fed (N = 5)	2.5% Cholesterol-fed (N = 4)
Deoxycholic	2.7 ± 1.3[a]	2.6 ± 1.1	2.9 ± 0.8
Chenodeoxycholic	8.1 ± 2.3	12.2 ± 2.5	15.4 ± 2.0
Hyodeoxycholic	6.3 ± 1.8	6.2 ± 2.8	5.8 ± 1.0
Cholic	18.1 ± 4.6	18.5 ± 4.7	18.4 ± 2.7
α-Muricholic	6.6 ± 2.1	5.9 ± 2.1	5.7 ± 0.1
β-Muricholic	1.8 ± 0.2	4.0 ± 1.0	8.0 ± 1.2[b]
Total	43.6 ± 6.0	49.4 ± 6.5	56.3 ± 3.8

[a]Means ± SEM.
[b]Significantly different from controls, $p < 0.05$.

TABLE 4

Diet Intake, Body Weight, Liver Weight and Cholesterol Levels in Three-day Postpartum Rats

Observations	Rat group: Control		0.5% Cholesterol-fed		2.5% Cholesterol-fed	
Diet intake (g/day)	14 ± 3[d]	(4)[e]	20 ± 4	(4)	20 ± 2	(6)
Body weight (g)	270 ± 9	(4)	286 ± 1.5	(4)	276 ± 5	(6)
Liver weight[a] (g)	11.1 ± 0.9	(4)	12.8 ± 0.9	(4)	12.8 ± 0.2	(6)
Serum cholesterol (mg/dl)						
Free	19.8 ± 6.1	(3)	18.5 ± 4.4	(3)	17.2 ± 4.0	(6)
Ester[b]	68.0 ± 8.7		58.5 ± 5.6		58.0 ± 6.8	
Total	87.8 ± 6.2	(3)	77.0 ± 3.4	(3)	75.2 ± 5.5	(6)
Hepatic cholesterol (mg/g liver[a])						
Free	0.44 ± 0.05[g]	(4)	1.11 ± 0.08[f]	(3)	0.64 ± 0.09	(4)
Ester	0.15 ± 0.02[g]	(4)	1.05 ± 0.06[f]	(3)	3.19 ± 0.38[f]	(4)
Total[c]	0.60 ± 0.05		2.16 ± 0.10[f]		3.83 ± 0.39[f]	

[a]Wet weight.
[b]The difference between total and free cholesterol.
[c]The sum of free and ester cholesterol.
[d]Means ± SEM.
[e]The number of samples studied.
[f]Significantly different from controls, $p < 0.05$.
[g]Significantly different from 20-day pregnant controls (cf. Table 1), $p < 0.05$.

Table 4 shows the daily food intake, body weight, liver weight and hepatic and serum levels of cholesterol in the three-day postpartum rats. Food intake, body weight and liver weight in either of the cholesterol-fed groups did not differ significantly from those in the control group. Cholesterol feeding caused no significant changes in the serum level of any cholesterol fraction. Esterified cholesterol was accumulated in the liver in both cholesterol-fed groups. In the 2.5% cholesterol-fed group, the free fraction also was accumulated. When the rats were fed the basal diet, the total cholesterol in the postpartum rats was lower than in the pregnant control rats owing to a decrease in the free cholesterol fraction (Tables 1 and 4).

Table 5 shows the composition of the bile acid pool in the postpartum rats. In the 2.5% cholesterol-fed group, the amount of lithocholic acid was greater but that of α-muricholic acid and β-muricholic acid was smaller than in the control group. The 0.5% cholesterol diet did not affect the bile acid composition significantly. In the control group, less lithocholic acid and more β-muricholic acid were found in the postpartum rats than in the pregnant rats (Tables 2 and 5).

The hepatic content of bile acids is shown in Table 6. The content of β-muricholic acid was higher in the 0.5% cholesterol-fed group than in the control group; 2.5% cholesterol feeding induced an accumulation of chenodeoxycholic acid and cholic acid ($p < 0.10$). Consequently, the total amount of bile acids was increased. Nevertheless, hepatic bile acids accounted for only 5% of the total pool in the body, as was the case for the control group (data not shown).

Metabolism of cholesterol and bile acids in fetal and neonatal rats. Table 7 shows body weight, liver weight, and cholesterol levels in liver and serum for 20-day fetal rats. Cholesterol feeding to the dam from the 11th to the 20th day of pregnancy did not affect the body weight or the liver weight in the 20-day fetal rats. Their serum cholesterol level also was unaffected by maternal cholesterol feeding. Hepatic total cholesterol level was, however, higher in both cholesterol-fed groups than in the control group owing to an increase in the free fraction.

Figure 1 shows typical gas chromatograms of methyl ester acetates of bile acids isolated from fetal pooled samples of the liver and of the gastrointestinal tract in the control group.

Table 8 shows the composition of the bile acid pool in the fetal rats. The content of chenodeoxycholic acid, α-muricholic acid ($p < 0.20$) and total bile acids was higher in the 0.5% cholesterol-fed group than in the control group. The content of chenodeoxycholic acid ($p < 0.10$) and total bile acids also was higher in the 2.5% cholesterol-fed group than in the control group.

The hepatic content of bile acids is shown in Table 9. Less cholic acid and β-muricholic acid ($p < 0.10$) were found in both cholesterol-fed groups than in the control group. There was a decrease in the hepatic content of total bile acids ($p < 0.20$) in the cholesterol-fed groups. In the control group, bile acids distributed in liver accounted for 37% of the total pool. The corresponding value for the 0.5% cholesterol-fed group was 22% and that for the 2.5% cholesterol-fed group 25% (data not shown).

Table 10 shows body weight, liver weight, and serum and liver cholesterol levels in the three-day neonatal rats. The body weight in both cholesterol-fed groups was

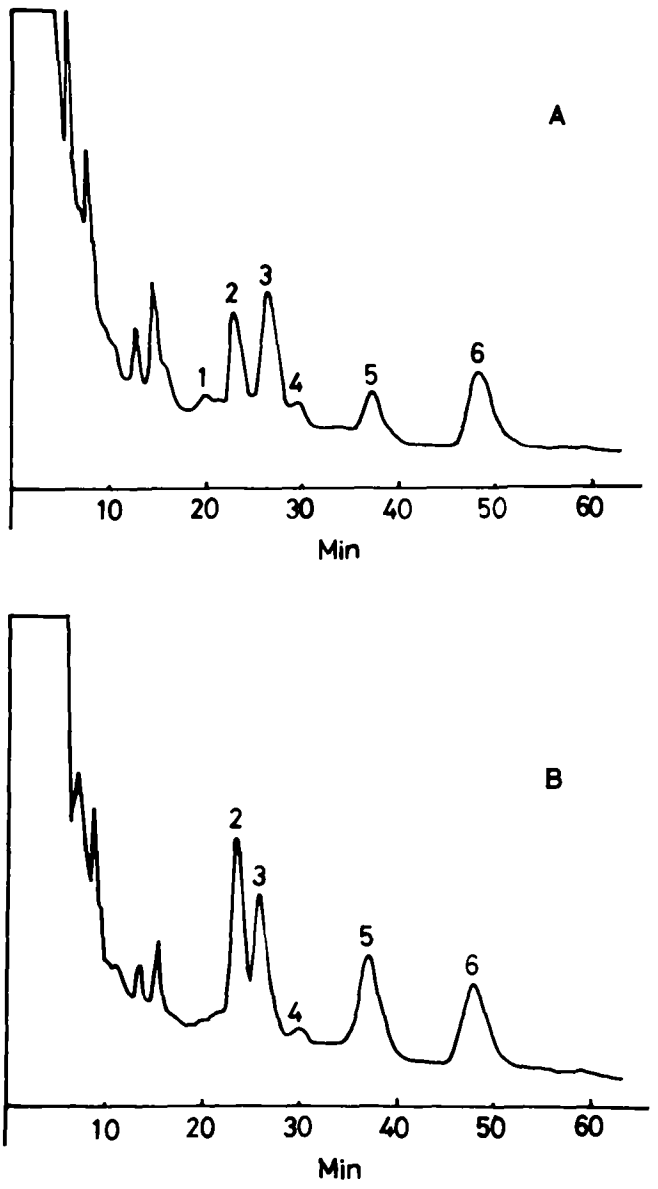

FIG. 1. Gas chromatograms of methyl ester acetates of bile acids isolated from the pooled samples of liver (A) and of gastrointestinal tract (B) in the control group of the 20-day fetal rats. The peaks identified were 1, deoxycholic acid; 2, chenodeoxycholic acid; 3, cholic acid; 4, hyodeoxycholic acid; 5, α-muricholic acid; 6, β-muricholic acid.

greater than that in the control group. There was no significant difference in liver weight between the control group and either cholesterol-fed group. The serum cholesterol level in the three-day neonatal rats was not affected by cholesterol feeding to the dam. The hepatic content of total cholesterol in both cholesterol-fed groups was higher than that in the control group. This was due to an increase in the free fraction in the 0.5% cholesterol-fed group and of the free and esterified cholesterol in the 2.5% cholesterol-fed group. The hepatic content of free and total cholesterol was higher in the neonatal control group than in the fetal control group (Tables 7 and 10).

Table 11 shows the composition of the bile acid pool. The chenodeoxycholic acid content was greater in the

TABLE 5

Composition of Bile Acid Pool in Three-Day Postpartum Rats

Bile acid	Pool size (mg/100 g body weight)		
	Control (N = 4)	0.5% Cholesterol-fed (N = 5)	2.5% Cholesterol-fed (N = 6)
Lithocholic	0.06 ± 0.02[b,e]	0.14 ± 0.04	0.38 ± 0.05[d]
Deoxycholic	0.94 ± 0.21	0.73 ± 0.12	0.88 ± 0.15
Chenodeoxycholic	1.64 ± 0.40	1.01 ± 0.23	1.18 ± 0.11
Hyodeoxycholic	1.39 ± 0.45	1.21 ± 0.16	0.52 ± 0.08
Cholic	6.66 ± 0.60	8.38 ± 1.97	5.71 ± 1.17
α-Muricholic	1.53 ± 0.32	0.95 ± 0.06	0.80 ± 0.12[d]
β-Muricholic	0.35 ± 0.06[e]	0.49 ± 0.10	Trace[c,d]
ω-Muricholic	0.25 ± 0.06	0.64 ± 0.26	0.08 ± 0.02
Others[a]	0.50 ± 0.05	0.53 ± 0.07	0.53 ± 0.07
Total	13.32 ± 0.94	14.08 ± 2.20	10.09 ± 1.20

[a]The sum of keto bile acids and minor unidentified peaks.
[b]Means ± SEM.
[c]Less than 30 μg/100 g body weight.
[d]Significantly different from controls, $p < 0.05$.
[e]Significantly different from 20-day pregnant controls (cf. Table 2), p. < 0.05.

TABLE 6

Hepatic Bile Acids in Three-Day Postpartum Rats

Bile acid	Hepatic content (μg/g liver weight)		
	Control (N = 4)	0.5% Cholesterol-fed (N = 5)	2.5% Cholesterol-fed (N = 6)
Deoxycholic	2.2 ± 1.1[a]	1.6 ± 0.4	3.3 ± 1.3
Chenodeoxycholic	18.9 ± 6.4	10.3 ± 2.4	32.6 ± 1.7[c]
Hyodeoxycholic	10.6 ± 3.1	6.5 ± 1.0	12.2 ± 3.6
Cholic	30.5 ± 7.6	38.3 ± 5.8	52.7 ± 8.2
α-Muricholic	7.1 ± 2.9	4.6 ± 0.6	7.4 ± 1.7
β-Muricholic	Trace[b,d]	4.4 ± 1.1[c]	Trace
Total	69.3 ± 10.9	65.7 ± 6.5	108.2 ± 9.4[c]

[a]Means ± SEM.
[b]Less than 0.5 μg/g liver weight.
[c]Significantly different from controls, $p < 0.05$.
[d]Significantly different from 20-day pregnant controls (cf. Table 3), $p < 0.05$.

TABLE 7

Body Weight, Liver Weight and Cholesterol Levels in 20-Day Fetal Rats

Observations	Rat group		
	Control	0.5% Cholesterol-fed	2.5% Cholesterol-fed
Body weight (g)	4.0 ± 0.0[d] [49][e]	4.0 ± 0.1 [30]	4.3 ± 0.0 [57]
Liver weight[a] (g)	0.28 ± 0.02 (6)[f]	0.29 ± 0.02 (3)	0.31 ± 0.02 (5)
Serum cholesterol (mg/dl)			
Free	45.0 ± 6.8 (4)	46.3 ± 4.8 (3)	44.5 ± 4.8 (4)
Ester[b]	75.0 ± 11.7	70.1 ± 10.3	79.5 ± 7.9
Total	120.0 ± 9.5 (4)	116.4 ± 9.1 (3)	124.0 ± 6.3 (4)
Hepatic cholesterol (μg/g liver[a])			
Free	77.4 ± 5.6 (4)	149.4 ± 11.2[g] (3)	130.2 ± 2.9[g] (4)
Ester	14.3 ± 0.9 (4)	17.5 ± 2.3 (3)	18.0 ± 1.4 (4)
Total[c]	91.3 ± 5.7	166.9 ± 11.4[g]	148.2 ± 3.2[g]

[a]Wet weight.
[b]The difference between total and free cholesterol.
[c]The sum of free and ester cholesterol.
[d]Means ± SEM.
[e]Figures in square brackets indicate the number of fetuses studied.
[f]Numbers in parentheses indicate the number of samples studied.
[g]Significantly different from controls, $p < 0.05$.

TABLE 8

Composition of Bile Acid Pool in 20-Day Fetal Rats

Bile acid	Pool size (μg/g body weight) Control (N = 5)	0.5% Cholesterol-fed (N = 6)	2.5% Cholesterol-fed (N = 7)
Deoxycholic	0.21 ± 0.03[a]	0.12 ± 0.04	0.13 ± 0.01
Chenodeoxycholic	1.89 ± 0.29	3.38 ± 0.34[b]	3.04 ± 0.43
Hyodeoxycholic	0.24 ± 0.05	0.33 ± 0.06	0.33 ± 0.06
Cholic	1.86 ± 0.36	2.49 ± 0.38	2.45 ± 0.33
α-Muricholic	1.42 ± 0.29	2.18 ± 0.28	2.05 ± 0.42
β-Muricholic	2.04 ± 0.36	2.78 ± 0.31	2.21 ± 0.22
Total	7.66 ± 0.65	11.28 ± 0.66[b]	10.21 ± 0.72[b]

[a]Means ± SEM.

[b]Significantly different from controls, $p < 0.05$.

TABLE 9

Hepatic Bile Acids in 20-Day Fetal Rats

Bile acid	Hepatic content (μg/g liver weight) Control (N = 5)	0.5% Cholesterol-fed (N = 6)	2.5% Cholesterol-fed (N = 7)
Deoxycholic	1.68 ± 0.21[a]	1.04 ± 0.24	0.91 ± 0.07
Chenodeoxycholic	9.70 ± 0.71	11.33 ± 0.87	11.83 ± 0.95
Hyodeoxycholic	1.24 ± 0.34	1.56 ± 0.18	1.21 ± 0.19
Cholic	9.88 ± 1.05	6.39 ± 0.59[b]	6.37 ± 0.86[b]
α-Muricholic	5.45 ± 1.01	5.02 ± 1.20	5.34 ± 1.00
β-Muricholic	16.45 ± 1.50	13.00 ± 1.06	12.98 ± 1.10
Total	44.40 ± 2.24	38.34 ± 2.18	38.64 ± 1.97

[a]Means ± SEM.

[b]Significantly different from controls, $p < 0.05$.

TABLE 10

Body Weight, Liver Weight and Cholesterol Levels in Three-Day Neonatal Rats

Observations	Rat group Control	0.5% Cholesterol-fed	2.5% Cholesterol-fed
Body weight (g)	5.8 ± 0.1[d] [50][e]	8.7 ± 0.2[g] [43]	8.2 ± 0.1[g] [48]
Liver weight[a] (g)	0.22 ± 0.22 (4)[f]	0.33 ± 0.02 (4)	0.32 ± 0.01 (6)
Serum cholesterol (mg/dl)			
Free	48.3 ± 17.3 (3)	52.3 ± 3.9 (4)	53.7 ± 6.4 (6)
Ester[b]	86.5 ± 20.6	92.9 ± 13.0	97.3 ± 11.6
Total	134.8 ± 11.1 (3)	145.2 ± 12.4 (4)	151.0 ± 9.7 (6)
Hepatic cholesterol (μg/g liver[a])			
Free	134.2 ± 22.4[h] (3)	504.6 ± 35.2[g] (4)	314.4 ± 3.3[g] (4)
Ester	20.5 ± 2.0 (3)	15.1 ± 0.4 (4)	322.5 ± 21.7[g] (4)
Total[c]	154.7 ± 22.5[h]	519.7 ± 35.2[g]	636.9 ± 21.9[g]

[a]Wet weight.

[b]The difference between total and free cholesterol.

[c]The sum of free and ester cholesterol.

[d]Means ± SEM.

[e]Figures in square brackets indicate the number of neonates studied.

[f]Numbers in parentheses indicate the number of sample studied.

[g]Significantly different from controls, $p < 0.05$.

[h]Significantly different from 20-day fetal controls (cf. Table 7), $p < 0.05$.

TABLE 11

Composition of Bile Acid Pool in Three-Day Neonatal Rats

Bile acid	Pool size (μg/g body weight)		
	Control (N = 6)	0.5% Cholesterol-fed (N = 6)	2.5% Cholesterol-fed (N = 6)
Chenodeoxycholic	1.67 ± 0.30[a]	1.73 ± 0.21	2.91 ± 0.31[b]
Hyodeoxycholic	0.30 ± 0.07	0.18 ± 0.00	0.15 ± 0.06
Cholic	100.86 ± 6.37[c]	90.65 ± 2.97	107.83 ± 8.24
α-Muricholic	1.03 ± 0.13	1.32 ± 0.15	1.65 ± 0.36
β-Muricholic	10.48 ± 1.41[c]	7.48 ± 0.79	6.13 ± 0.78[b]
Total	114.34 ± 6.54[c]	101.36 ± 3.09	118.14 ± 8.29

[a]Means ± SEM.

[b]Significantly different from controls, $p < 0.05$.

[c]Significantly different from the controls of 20-day fetal rats (cf. Table 8), $p < 0.05$.

TABLE 12

Hepatic Bile Acids in Three-Day Neonatal Rats

Bile acid	Hepatic content (μg/g liver weight)		
	Control (N = 6)	0.5% Cholesterol-fed (N = 6)	2.5% Cholesterol-fed (N = 6)
Chenodeoxycholic	3.58 ± 0.32[a]	2.42 ± 0.32[b]	2.67 ± 0.24[b]
Hyodeoxycholic	8.40 ± 1.01	4.89 ± 0.05	3.87 ± 1.04
Cholic	49.08 ± 2.21	55.98 ± 3.07	55.34 ± 2.77
α-Muricholic	2.87 ± 0.19	4.83 ± 0.70	2.71 ± 0.47
β-Muricholic	11.82 ± 1.48	19.65 ± 1.61	22.37 ± 2.32[b]
Total	75.75 ± 2.67	87.77 ± 3.55[b]	86.96 ± 3.80

[a]Means ± SEM.

[b]Significantly different from controls, $p < 0.05$.

2.5% cholesterol-fed group than in the control group, but the β-muricholic acid content was lower in the 2.5% cholesterol-fed group (0.5% cholesterol-fed group, $p < 0.10$) than in the control group. The bile acids in the neonatal rats were composed mainly of cholic acid, and the pool size of cholic acid was greatly expanded as compared with that in the 20-day fetal rats (Tables 8 and 11). These results were similar to those reported by Little et al. (9).

The hepatic content of bile acids is shown in Table 12. In the cholesterol-fed groups, there was a decrease in chenodeoxycholic acid but an increase in β-muricholic acid as compared with the control group. Only 2-3.5% of the bile acids in the total pool were found in the liver in all the groups of the neonatal rats (data not shown).

DISCUSSION

Cholesterol feeding induces accumulation of cholesterol in the liver without affecting the serum cholesterol level in the adult rats (18,19). The present results show that cholesterol feeding to the dams affects hepatic cholesterol metabolism not only in the dam but also in the pup. The level of free cholesterol in the rat liver remained within a relatively narrow range, whereas the amount of esterified cholesterol increased during cholesterol feeding. Hepatic esterified cholesterol is formed in situ by acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26). Its activity can be altered by hepatic cholesterol content (20,21). Some reports have shown that by feeding pregnant rats a cholesterol-supplemented diet, cholesterol synthesis is reduced in the maternal liver but not in the fetal liver (7,22). In our experiments, the amount of cholesterol accumulated in liver may have been insufficient for inducing the activity of the cholesterol-esterifying enzyme in the fetus and the neonate. In the neonate of the 2.5% cholesterol-fed dam, however, the accumulated cholesterol in the liver might be sufficient for increasing the ability of esterification.

The resulting increase in hepatic cholesterol can affect bile acid metabolism. Wilson (23) demonstrated clearly that cholesterol feeding causes a great increase in excretion of bile acids in the adult rats. In addition, cholesterol feeding leads to the increase in chenodeoxycholic acid, α-muricholic acid, β-muricholic acid and hyodeoxycholic acid in the pool with a concomitant decrease of cholic acid (1-3). Such a shift in the bile acid spectrum in the cholesterol-fed rats indicates that the activity of cholesterol 7α-hydroxylase (EC 1.14.13.17) was accelerated, and 6β-hydroxylation of chenodeoxycholic acid and lithocholic acid was stimulated; however, 12α-hydroxylation leading to the formation of cholic acid was suppressed (3). Beher

et al. (1,2,24) have shown that the half-life of chenodeoxycholic acid is shorter than that of cholic acid in this species. Therefore, the increased synthesis of chenodeoxycholic acid is postulated to be a protective mechanism for excreting the excess of dietary cholesterol (1,2). The increase in β-muricholic acid synthesis also may be one of several protective mechanisms since this acid is nontoxic and ineffective in inhibiting bile acid synthesis (25,26). The change in the metabolism of bile acids except for hyodeoxycholic acid in the pregnant rats appears to be a response to a high level of dietary cholesterol occurring in the same way as the well-documented response of bile acid metabolism in adult rats to a high cholesterol diet. Hyodeoxycholic acid has been shown to be derived from the products of chenodeoxycholic acid, or β-muricholic acid and lithocholic acid (27,28). However, the increase of chenodeoxycholic acid pool size did not always elevate the concentration of hyodeoxycholic acid in the pool in spite of the increase in lithocholic acid or β-muricholic acid (29,30).

In the postpartum rats, bile acid synthesis seems to be inhibited by the increase in hepatic chenodeoxycholic acid and/or cholic acid, especially in the 2.5% cholesterol-fed group. The increase of chenodeoxycholic acid or cholic acid content in the adult rats can inhibit the bile acid synthesis (30-33). Besides, the resulting decrease of 6β-hydroxylation of chenodeoxycholic acid or lithocholic acid is assumed to increase the amount of lithocholic acid. The hormonal changes occurred after delivery alternatively may induce the changes in the response of bile acid metabolism to high dietary cholesterol not seen in the pregnant rats. In this connection, Subbiah and Buscaglia (34) found that the cholic acid/chenodeoxycholic acid ratio in the bile of pregnant rats was lower than in the bile of virgin female rats.

A part of the bile acids in fetal rats have been thought to originate from the maternal transfer across the placenta (9,35,36). However, Naseem et al. (37) have reported that the activity of cholesterol 7α-hydroxylase in the fetal rats was increased by cholesterol feeding during pregnancy. Therefore, an increase of some bile acids in the fetal rats by cholesterol intake may result from the stimulation of bile acid synthesis in them. Naseem et al. (37) also showed that bile acid synthesis in the neonatal rats is facilitated in the same way as the fetal rats by cholesterol feeding during pregnancy. In addition, Sprinkle et al. (38) has shown that in the neonatal rats born from dams fed a chenodeoxycholic acid-supplemented diet during gestation the activity of hepatic cholesterol 7α-hydroxylase was not inhibited in spite of a significant increase in the chenodeoxycholic acid content in the neonatal liver. In our experiments, the increase of chenodeoxycholic acid in the neonatal rats was induced by cholesterol intake but β-muricholic acid synthesis was relatively suppressed; consequently, the total amount of bile acids was not significantly changed.

Although the effect of high cholesterol intake of the dams on bile acid metabolism in the fetal and the neonatal rats has not been identified clearly, the response of bile acid metabolism in the fetal and the neonatal rats to high cholesterol intake of the maternal rats may be independent of that in the dams. In contrast to rats, neonatal bile acid synthesis in guinea pigs is reduced by feeding cholesterol or chenodeoxycholic acid to the dam (39,40). It is not known if the difference between the rat and the guinea pig neonates is species-related, but it is known that guinea pigs do not respond well to dietary cholesterol (41).

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REFERENCES

1. Beher, W.T., Rao, B., Beher, B.E., Semnuk, G., and Vulpetti, N. (1967) *Henry Ford Hosp. Med. 15,* 107-118.
2. Beher, W.T., Casazza, K.K., Beher, M.E., Filu, A.M., and Bertasius, J. (1970) *Proc. Soc. Exp. Biol. Med. 134,* 595-602.
3. Gustafsson, B.E., Einarsson, K., and Gustafsson, J.-A. (1975) *J. Biol. Chem. 250,* 8496-8502.
4. Chevallier, E.T. (1964) *Biochim. Biophys. Acta 84,* 316-339.
5. Raphael, B.C., Patton, S., and McCarthy, R.D. (1975) *FEBS Lett. 58,* 47-49.
6. Clarenberg, R., and Chaikoff, I.L. (1966) *J. Lipid Res. 7,* 27-37.
7. Calandra, S., Quartaroli, G.C., and Montaguti, M. (1975) *Eur. J. Clin. Invest. 5,* 27-31.
8. Lester, R., Little, J.M., Greco, R., Piasecki, G.J., and Jackson, B.T. (1972) *Pediatr. Res. 6,* p. 375.
9. Little, J.M., Richey, J.E., Thiel, V.D.H., and Lester, R.J. (1979) *J. Clin. Invest. 63,* 1042-1049.
10. Hsia, S.L. (1971) in *The Bile Acids* (Nair, P.P., and Kritchevsky, D., eds.) Vol. 1, pp. 95-120, Plenum Press, New York, NY.
11. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
12. Ichida, T. (1963) *Hokkaido Igaku Zasshi 38,* 55-66.
13. Frosch, B., and Wagner, H. (1967) *Nature 213,* 404-405.
14. Okishio, T., Nair, P.P., and Gordon, M. (1967) *Biochem. J. 102,* 654-659.
15. Makino, I., and Sjövall, J. (1972) *Anal. Lett. 5,* 341-349.
16. Makino, I., Shinozaki, K., Nakagawa, S., and Mashimo, K. (1974) *J. Lipid Res. 15,* 132-138.
17. Tsuda, K., Sato, Y., Ikekawa, N., Tanaka, S., Higashikuze, H., and Okusawa, R. (1965) *Chem. Pharm. Bull. 13,* 720-723.
18. Ho, K.J. (1968) *Arch. Path. 86,* 585-596.
19. Mathe, D., and Chevallier, F. (1979) *J. Nutr. 109,* 2076-2084.
20. Spector, A.A., Mathur, S.N., and Kaduce, T.L. (1979) *Prog. Lipid Res. 18,* 31-53.
21. Suckling, K.E., and Strange, E.F. (1985) *J. Lipid Res. 26,* 647-671.
22. Feingold, K.R., Wiley, T., Moser, A.H., Lear, S.R., and Wiley, M.H. (1983) *J. Lab. Clin. Med. 101,* 251-263.
23. Wilson, J.D. (1964) *J. Lipid Res. 5,* 409-417.
24. Beher, W.T., Beher, M.E., and Rao, B. (1967) *Life Sci. 6,* 863-866.
25. Greim, H., Trülzsch, D., Roboz, J., Dressler, K., Czygan, P., Hutterer, F., Schaffner, F., and Popper, H. (1972) *Gastroenterology 63,* 837-845.
26. Kinugasa, T., Uchida, K., Kadowaki, M., Takase, H., Nomura, Y., and Saito, Y. (1981) *J. Lipid Res. 22,* 201-207.
27. Einarsson, K. (1966) *J. Biol. Chem. 241,* 534-539.
28. Heijenoort, Y.V., Sacquest, E., and Riottot, M. (1974) *Nutr. Metab. 17,* 65-73.
29. Sprinkle, D.J., Hassan, A.S., and Subbiah, M.T.R. (1984) *Proc. Soc. Exp. Biol. Med. 175,* 386-397.
30. Danielsson, H., and Johansson, G. (1974) *Gastroenterology 67,* 126-134.
31. Danielsson, H. (1973) *Steroids 22,* 667-675.
32. Shefer, S., Hauser, S., Lapar, B., and Mosbach, E.H. (1973) *J. Lipid Res. 14,* 573-580.
33. Gustafsson, B.E., Angelin, B., Björkhem, I., Einarsson, K., and Gustafsson, J.-A. (1981) *Lipids 16,* 228-233.
34. Subbiah, M.T.R. and Buscaglia, M.D. (1976) *Res. Commun. Chem. Pharmacol. 13,* 529-539.
35. Hassan, A.S., and Subbiah, M.T.R. (1980) *Steroids 36,* 709-715.
36. Hassan, A.S., and Subbiah, M.T.R. (1980) *Proc. Soc. Exp. Biol. Med. 165,* 490-495.
37. Naseem, S.M., Khan, M.A., Heald, F.P. and Nair, P.P. (1980) *Atherosclerosis 36,* 1-8.

38. Sprinkle, D.J., Hassan, A.S., and Subbiah, M.T.R. (1984) *Proc. Soc. Exp. Biol. Med. 175,* 386-397.
39. Hassan, A.S., Yunker, R.L., and Subbiah, M.T.R. (1981) *J. Nutr. 111,* 2030-2033.
40. Hassan, A.S., Yunker, R.L., Sprinkle, D.J., and Subbiah, M.T.R. (1983) *J. Nutr. 113,* 986-995.
41. Traber, M.G., and Ostwald, R. (1978) *J. Lipid Res. 19,* 448-456.

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Linoleic Acid Requirement of Rats Fed *trans* Fatty Acids

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The amount of linoleic acid required to prevent undesirable effects of C18 *trans* fatty acids was investigated. In a first experiment, six groups of rats were fed diets with a high content of *trans* fatty acids (20% of energy [en%]), and increasing amounts of linoleic acid (0.4 to 7.1 en%). In a second experiment, four groups of rats were fed diets designed to compare *trans* fatty acids with saturated and *cis*-monounsaturated fatty acids of the same chain length at the 2 en% linoleic acid level. After 9-14 weeks, the oxygen uptake, lipid composition and ATP synthesis of heart and liver mitochondria were determined.

The phospholipid composition of the mitochondria did not change, but the fatty acid compositions of the two main mitochondrial phospholipids were influenced by the dietary fats. *Trans* fatty acids were incorporated in all phospholipids investigated. The linoleic acid level in the phospholipids, irrespective of the dietary content of linoleic acid, increased on incorporation of *trans* fatty acids. The arachidonic acid level had decreased in most phospholipids in animals fed diets containing 2 en% linoleic acid. At higher linoleic acid intakes, the effect of *trans* fatty acids on the phospholipid arachidonic acid level diminished. However, in heart mitochondrial phosphatidylethanolamine, *trans* fatty acids significantly increased the arachidonic acid level. Despite these changes in composition, neither the amount of dietary linoleic acid nor the addition of *trans* fatty acids influenced the mitochondrial function. For rats, a level of 2 en% of linoleic acid is sufficient to prevent undesirable effects of high amounts of dietary C18 *trans* fatty acids on the mitochondrial function.
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Trans fatty acids from partially hydrogenated oils and from ruminant fats form part of the human diet. Throughout the years, there has been a marked interest in the biological effects of *trans* fatty acids. It now is recognized that they are well-absorbed by both man and experimental animals, and that they are found in most tissues (1-5). *Trans* fatty acids are incorporated into triacylglycerol and phospholipids of biological membranes (1,2,6). The effect of the incorporation of *trans* fatty acids on the biological function of membranes is an unanswered question.

Many investigators have used mitochondria to investigate the effects of dietary fatty acids on biological membranes (7-14). Mitochondrial oxygen uptake and ATP synthesis are regarded as sensitive markers for the functionality of the mitochondria. Changes in the fatty acid composition of mitochondria were shown to cause diminished mitochondrial function in rats fed diets containing erucic acid (9,15,16). The amount of essential fatty acids in the diet also affected the mitochondrial composition and/or function (7,11,12). The amount of *trans* fatty acids incorporated into mitochondrial membranes may be as high as 17% of the total fatty acid content (17).

Trans fatty acids formed during the partial hydrogenation of vegetable oils appear not to have undesirable effects when compared with saturated or *cis*-monounsaturated fatty acids, provided sufficient linoleic acid is present in the diet (1-3,6,18). It was our aim to quantify the amount of linoleic acid necessary.

In a first experiment, we investigated the effects of diets having a constant amount of C18 *trans* fatty acids and an increasing amount of linoleic acid on the mitochondrial composition and function. In the second, we compared the effects of *trans* fatty acids with those of long chain (C16,C18) saturated and *cis*-monounsaturated fatty acids at a linoleic acid level of 2 en%.

EXPERIMENTAL

Materials. Partially hydrogenated soybean oil (PHSO) was specially prepared by Van den Bergh and Jurgens (Rotterdam, The Netherlands); hydrogenated coconut oil (HCNO) from Chempry BV (Raamsdonkveer, The Netherlands), sunflower seed oil (SSO) from Union (Antwerpen, Belgium) and cocoa butter (CB) from J. Schoenmaker BV (Zaandam, The Netherlands). Olive oil (OV) was purchased from Fol Jr & Co. (Krimpen aan de IJssel, The Netherlands) and the low-linoleic acid olive oil (OV-LL) was a gift from Elais (Greece). Bovine serum albumin (fraction V, essentially free from fatty acid), L-glutamate, and DL-malate were from Sigma Chemical Co. (St. Louis, MO); ADP and ATP were from Boehringer (Mannheim, FRG), Nagarose from *Bacillus amyloboliquefaciens* was from Serva (Heidelberg, FRG), ethylenediamine tetraaceticacid (EDTA) (p.a.), diethyl ether and hexane were from Baker Chemicals (Deventer, The Netherlands), sucrose was from BDH Chemicals Ltd. (Poole, England), 2,6-di-*tert*-butyl-*p*-cresol (BHT) was from Fluka AG (Buchs, Switzerland). The HPTLC plates (Silica gel F254), and all other reagents were from Merck (Darmstadt, FRG).

Animals and diets. Weanling male SPF-Wistar rats (CPB=WU, Central Breeding Station TNO Zeist, The Netherlands) were used in both experiments. The animals were housed individually in a climatized room. The mean temperature was 23 C, the relative humidity was 45-70%, and there was a day/night cycle of 12/12 hr. The animals had free access to water and food. This food, a semisynthetic diet with 40 en% fat, was composed of (in $g.MJ^{-1}$) casein, 14.8; vitamin mixture, 0.2; salt mixture, 1.3; cellulose, 3.8; maize starch, 25.2; experimental fat, 10.3 (for composition of vitamin and salt mixture see ref. 19). The animals were weighed and examined weekly.

For the first experiment, PHSO, HCNO, OV and SSO were mixed to yield six diets having a graduated linoleic acid content (0.4-7.1 en%) but a constant *trans* fatty acids

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Abbreviations: ADP/O, number of ADP mol converted into ATP per mol O; BHT, 2,6-di-*tert*-butyl-*p*-cresol; CB, cocoa butter; CL, cardiolipin; EDTA, ethylenediamine tetraacetic acid; GC, gas chromatography; HCNO, hydrogenated coconut oil; OV, olive oil; OV-LL, low-linoleic olive oil; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PHSO, partially hydrogenated soybean oil; PI, phosphatidylinostol; PS, phosphatidylserine; QO_2, oxygen consumption; rATP, rate of ATP synthesis; RCR, respiratory control ratio; S, sphingomyelin; SSO, sunflower seed oil; TLC, thin layer chromatography.

TABLE 1

Composition (en%) of Experimental Fats and Calculated Linoleic Acid Content (en%) in the Final Diet

Dietary oil[a]	Experimental fats							
	1	2	3	4	5	6	7[b]	8[b]
PHSO	30.0	30.0	30.0	30.0	30.0	30.0	—	—
HCNO	7.3	5.0	5.0	5.0	3.0	—	22.6	—
OV	2.7	4.8	2.7	1.0	—	—	17.4	39.3
SSO	—	0.2	2.3	4.0	7.0	10.0	—	0.7
LA[c]	0.4	0.8	2.0	3.0	5.0	7.1	2.0	5.0

[a]PHSO, partially hydrogenated soybean oil; HCNO, hydrogenated coconut oil; OV, olive oil; SSO, sunflower seed oil.

[b]References.

[c]Linoleic acid.

TABLE 2

Calculated Fatty Acid Composition (%) of the Experimental Fats (Groups), First Experiment

Type of fatty acid[a]	Group							
	1	2	3	4	5	6	7[b]	8[b]
6:0	0.1	—	—	—	—	—	0.2	—
8:0	1.2	0.8	0.8	0.8	0.5	—	3.8	—
10:0	1.0	0.7	0.7	0.7	0.4	—	3.2	—
12:0	8.9	6.1	6.1	6.1	3.7	0.1	27.0	—
14:0	3.6	2.5	2.5	2.5	1.6	0.2	10.3	—
16:0	10.3	10.4	10.1	9.9	9.6	9.4	10.5	12.2
16:1	0.3	0.3	0.3	0.2	0.2	0.2	0.4	1.0
17:0	0.1	0.1	0.1	0.1	0.1	0.2	—	0.1
17:1	—	—	—	—	—	0.1	0.1	0.2
18:0	8.5	7.9	8.1	8.1	7.8	7.2	8.0	2.9
18:1 *c*	16.5	20.3	17.5	15.3	14.8	16.1	30.7	69.7
18:1 *t*	39.1	39.1	39.1	39.1	39.1	39.1	—	—
18:2 9*c*, 12*c*	1.0	2.0	5.0	7.0	12.5	17.7	5.0	12.5
18:2 *ct,tc* 9,12	0.6	0.6	0.6	0.6	0.6	0.6	—	—
18:2 9*t*,12*t*	0.5	0.5	0.5	0.5	0.5	0.5	—	—
18:2 other	7.7	7.7	7.7	7.7	7.7	7.7	—	—
18:3	—	—	—	—	—	0.1	0.2	0.5
20:0	0.3	0.3	0.3	0.3	0.4	0.4	0.2	0.2
20:1[c]	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.3
22:0	0.3	0.4	0.3	0.3	0.4	0.5	0.1	0.3
Actual LA-level[d] (%)	1.1	n.d.	5.4	n.d.	n.d.	n.d.	5.2	n.d.

[a]The notation for the fatty acid indicates chain length and number of double bonds: *c*, *cis*-isomers; *t*, *trans*-isomers.

[b]References.

[c]Also contains conjugated 18:2 isomers.

[d]Determined by GLC.

n.d., Not determined.

level of 20 en% (Table 1). Two fats served as references, one consisting of HCNO and OV (2 en% linoleic acid), another consisting mainly of OV (5 en% linoleic acid). Table 2 shows the calculated fatty acid compositions of the experimental fats. Seven groups consisted of 12 animals each and one of 24 animals: group 3. The rats were fed the experimental diets for 13–14 wk.

In the second experiment, four groups of 24 rats each (randomly selected from 24 litters of four rats) were fed diets containing either 20 en% *trans* fatty acid, PHSO,

or mainly saturated fatty acids, CB, or *cis*-monounsaturated fatty acids, OV-LL, or a mixture of PHSO, CB and OV (PHSO mix, 10 en% *trans* fatty acids, see Table 3). All diets contained 2 en% linoleic acid and were fed for 9–11 weeks. The *(trans)* fatty acid compositions are given in Table 4.

Analytical: fatty acid composition of dietary fats. The composition of PHSO was determined by a combination of $AgNO_3$-thin layer chromatography (TLC) separation and capillary gas chromatography (GC). Fatty acid methyl esters were prepared by methylation of fatty acids with BF_3 and subsequent separation on precoated thin-layer SiO_2-plates (impregnated with $AgNO_3$) by elution with toluene. The fatty acid methyl ester fractions were analyzed according to Scholfield (20). The compositions of the other fats and oils were determined by GLC (5% DEGS packed column) after *trans*-methylation with methanolic HCl.

Mitochondrial oxygen uptake and ATP synthesis. At the end of the feeding period, the animals were fasted overnight, weighed and killed by aorta cannulation under ether anesthesia. Heart and liver were removed immediately and washed in an ice-cold buffer. The liver mitochondria were prepared essentially as described by Clouet and Bezard (21). The heart mitochondria were prepared according to Hülsmann (22). The mitochondrial respiration was measured according to Swarttouw (23) using a Clark oxygen electrode (Gilson Oxygraph, Gilson Electronics, Middleton, WI). Glutamate and malate were used as substrate. The reaction was started by adding ADP. The state-3 respiratory rate in the presence of ADP, the state-4 respiration after exhaustion of ADP, the respiratory control ratio, the ADP/O ratio, and the ATP synthesis rate were calculated as described by Estabrook (24).

Lipid composition of mitochondrial membranes. Immediately after isolation and addition of BHT, part of the mitochondria were frozen and stored at −25 C. Before analysis, six samples within each group were pooled. Lipid was extracted according to Bligh and Dyer (25). The phospholipid fraction of the liver mitochondria (first experiment) was isolated on a silica gel column; the phospholipid classes were separated by HPLC according to Patton et al. (26). The phospholipid fraction of the heart mitochondria (both experiments) was isolated on TLC. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), cardiolipin (CL) and sphingomyelin (S) were isolated by TLC according to Christie (27). The phospholipids were quantitated as described by Bartlett (28). The fatty acids of PC and PE were *trans*-methylated with methanolic HCl and analyzed by GLC using a packed (5% DEGS) column or a glass capillary column (Silar 88). The fatty acid distribution was expressed as area percentage. Because the fatty acid analyses were performed on two or four pooled samples of six animals each, only the means and no standard errors of the means are given.

Statistical. The mitochondrial respiration data were subjected to an analysis of variance according to the randomized complete blocks design. Days of killing served as blocks in the first experiment, litters in the second. In the first experiment, Dunnett's procedure was applied to find which groups differed systematically from the standard group (group 3, 20 en% *trans* fatty acids and 2 en% linoleic acid). In the second experiment, the Student-Newman-Keuls multiple-range test was applied to locate possible systematic differences between the dietary treatments.

TABLE 3

Composition (en%) of Experimental Fats (Groups), Second Experiment

Dietary oils[a]	Experimental fats			
	CB	OV	PHSO	PHSOMix
PHSO	—	—	30.0	15.0
OV-LL	—	40.0	—	—
OV	—	—	2.7	15.0
CB	39.4	—	—	10.0
SSO	0.6	—	2.3	—
HCNO	—	—	5.0	—

[a]PHSO, partially hydrogenated soybean oil; OV-LL, low-linoleic acid olive oil; OV, olive oil; CB, cocoa butter; SSO, sunflower seed oil; HCNO, hydrogenated coconut oil.

TABLE 4

Fatty Acid Compositions (%) of the Experimental Fats (Groups), Second Experiment

Type of fatty acid[a]	Group			
	CB	OV	PHSO	PHSOMix
6:0	—	—	—	—
8:0	—	—	0.8	—
10:0	—	—	0.7	—
12:0	—	0.1	5.6	—
14:0	0.2	—	2.4	0.1
16:0	23.5	11.9	10.0	15.3
16:1	—	—	0.3	—
17:0	0.3	—	0.1	0.1
17:1	—	0.1	0.1	0.1
18:0	31.9	2.0	8.0	12.1
18:1 *c*	36.9	78.7	17.5	39.9
18:1 *t*	—	—	39.1	19.5
18:2 9*c*,12*c*	4.5	5.5	5.3	4.7
18:2 *ct*,*tc* 9,12	—	—	0.6	0.3
18:2 9*t*,12t	—	—	0.5	0.2
18:2 other	—	—	7.7	3.9
18:3	0.2	0.6	—	—
20:0	1.3	0.4	0.5	0.6
20:1	—	0.4	0.5[b]	0.4[b]
22:0	0.3	0.2	0.4	0.3

[a]The notation indicates chain length and number of double bonds. *c*, *cis*-isomer; *t*, *trans*-isomer.

[b]Also contains conjugated 18:2 isomers.

RESULTS

General condition of the animals. All animals were in good health in both experiments, showing no overt signs of EFA deficiency. Macroscopic examination of the animals did not reveal treatment-related differences. Systematic differences in food consumption or body weight related to dietary treatment did not occur in either experiment. At the time of killing, the weight of the animals ranged

TABLE 5

Oxygen Uptake and Rate of ATP-Synthesis of Rat Heart and Liver Mitochondria, First Experiment[a]

Group	TFA[c] (en%)	Heart				Liver			
		QO_2[d]	*r*ATP[e]	ADP/O *f*	RCR*g*	QO_2[d]	*r*ATP[e]	ADP/O *f*	RCR*g*
1	20	371 ± 10	2038 ± 50	2.75 ± 0.025	6.4 ± 0.44	68 ± 2.9	287 ± 17	2.10 ± 0.059	2.2 ± 0.13
2	20	365 ± 18	1993 ± 93	2.74 ± 0.025	6.2 ± 0.45	64 ± 3.2[h]	263 ± 18[h]	2.04 ± 0.055[i]	2.1 ± 0.20[i]
3	20	347 ± 14	1901 ± 79	2.73 ± 0.027	6.5 ± 0.43	76 ± 4.1	338 ± 24	2.21 ± 0.052	2.5 ± 0.15
4	20	373 ± 16	2032 ± 82	2.73 ± 0.032	6.7 ± 0.50	77 ± 3.1	344 ± 16	2.22 ± 0.048	2.6 ± 0.16
5	20	369 ± 19	2027 ± 101	2.75 ± 0.026	6.5 ± 0.56	75 ± 4.9	336 ± 23	2.24 ± 0.028	2.6 ± 0.13
6	20	378 ± 17	2071 ± 94	2.74 ± 0.030	6.6 ± 0.46	68 ± 3.4	300 ± 18	2.20 ± 0.043	2.5 ± 0.12
7[b]	0	366 ± 17	2006 ± 88	2.75 ± 0.023	6.4 ± 0.47	71 ± 3.3	310 ± 17	2.17 ± 0.034	2.5 ± 0.11
8[b]	0	354 ± 19	1933 ± 103	2.73 ± 0.033	6.3 ± 0.49	72 ± 3.5	301 ± 20	2.20 ± 0.050	2.7 ± 0.16

[a]Each value represents the mean value ± s.e.m. of 12 animals.
[b]References.
[c]TFA, *trans* fatty acids.
[d]QO_2, oxygen consumption (nmol.min^{-1}.mg^{-1} protein).
[e]*r*ATP, rate of ATP-synthesis (nmol.min^{-1}.mg^{-1} protein).
[f]ADP/O, mol ADP converted into ATP per mol O.
[g]RCR, respiratory control ratio.
[h,i], significantly different from values of group 3: $P < 0.1$ and $P < 0.05$, respectively.

from 370–400 g in the first experiment and from 350–400 g in the second.

Oxygen uptake and ATP-synthesis in mitochondria. The data on the heart mitochondria, i.e. oxygen consumption (QO_2), number of ADP mol converted into ATP per mol O (ADP/O), rate of ATP-synthesis (*r*ATP) and respiratory control ratio (RCR), have been depicted in Table 5 (first experiment). No significant differences, induced by the dietary treatments, could be detected. Even at a very low linoleic acid intake (0.4 en%), the functionality of the mitochondria was not impaired. The respiratory capacity of the liver mitochondria was also analyzed (Table 5). In the liver mitochondria of group 2 (0.8 en% linoleic acid), the QO_2, *r*ATP, ADP/O and RCR decreased significantly compared to group 3 (2.0 en% linoleic acid). There were no significant differences between group 3 (20 en% *trans* fatty acids and 2 en% linoleic acid) and the reference groups 7 and 8. Data on the respiratory capacity of the heart mitochondria (second experiment) are given in Table 6. No significant differences were detected between any of the groups.

Phospholipid composition of mitochondria: heart. There was some variation between the groups in the phospholipid composition of the heart mitochondria of the first experiment (Table 7), although no trends in phospholipid levels induced by the dietary linoleic acid could be detected. No effect of *trans* fatty acids on phospholipid distribution was observed. In the second experiment, a similar distribution of phospholipids in the mitochondria was found (Table 8). The differences in the phospholipid composition (mainly that of the minor phospholipids) in both experiments probably were caused by slight differences in the preparation procedure of the mitochondria and by differences in the phospholipid analysis. Since reliable values for phospholipids other than PC cannot be given, the phospholipid composition of the CB group is incomplete. Only minor differences between the groups can be detected. The differences between the groups fed diets containing *trans* fatty acids (PHSO and PHSOMix) were in the same order of magnitude or even greater than those between groups fed *trans* fatty acids and those fed other diets, indicating that no changes related to *trans* fatty acid were induced.

TABLE 6

Oxygen Uptake and Rate of ATP Synthesis of Rat Heart Mitochondria, Second Experiment[a]

Group	Parameter			
	QO_2[b]	*r*ATP[c]	ADP/O[d]	RCR[e]
CB	50 ± 2.9	261 ± 16	2.63 ± 0.067	4.8 ± 0.37
OV	50 ± 4.1	256 ± 23	2.63 ± 0.088	5.5 ± 0.44
PHSO	53 ± 3.7	283 ± 23	2.69 ± 0.103	4.9 ± 0.35
PHSOMix	49 ± 3.4	268 ± 22	2.80 ± 0.113	5.3 ± 0.40

[a]Each value represents the mean value ± s.e.m. of 24 animals.
[b]QO_2, oxygen consumption (nmol.min^{-1}.mg^{-1} protein).
[c]*r*ATP, rate of ATP-synthesis.
[d]ADP/O, mol ADP converted into ATP per mol O.
[e]RCR, respiratory control ratio.

Liver. The phospholipid composition of the liver mitochondria was somewhat different from that of the heart mitochondria (Table 7): their PC level was higher, whereas their cardiolipin level was lower. Increasing linoleic acid intake increased the fraction of PC of the phospholipids at the expense of PE. No effects of *trans* fatty acids were observed.

Fatty acid composition of phosphatidylcholine: heart. Saturated and monounsaturated fatty acids hardly were influenced by the amount of linoleic acid in the diet except for a slight decrease in the 18:1 level with increased

TABLE 7

Phospholipid Composition (%) of Rat Heart and Liver Mitochondria, First Experiment[a]

Organ	Group	Phospholipids					
		PC[b]	PE[c]	PI[d]	PS[e]	CL[f]	S[g]
Heart	1	33.2	25.4	11.0	8.3	12.6	4.3
	2	34.4	27.3	12.8	10.2	10.5	4.1
	3[h]	34.6	29.6	10.0	7.0	14.3	4.0
	4	31.8	28.6	9.8	4.5	15.8	4.2
	5	33.8	28.2	10.0	7.8	14.1	6.2
	6	32.6	28.2	11.8	8.5	14.3	4.4
	7[i]	34.5	31.2	7.5	4.2	15.8	3.8
	8[i]	32.3	27.6	12.9	7.8	15.6	4.5
				PI + PS			
Liver	1	42	38	14		6	—
	2	48	35	12		5	—
	3[h]	49	34	12		5	—
	4	48	33	13		6	—
	5	58	29	9		3	—
	6	49	34	13		4	—
	7[i]	45	37	12		7	—
	8[i]	50	33	13		4	—

[a]Each value represents the mean of two pools of six animals.
[b]PC, phosphatidylcholine.
[c]PE, phosphatidylethanolamine.
[d]PI, phosphatidylinositol.
[e]PS, phosphatidylserine.
[f]CL, cardiolipin.
[g]S, sphingomyelin.
[h]Mean of four pools of six animals.
[i]References.
—, Not determined.

linoleic acid intake (first experiment; Table 9). Surprisingly, the 18:2 level (mainly linoleic acid) remained rather constant despite its wide range of dietary levels (0.4–7.1 en%). With an increasing linoleic acid intake, the level of arachidonic acid increased to about 34%, then it remained constant; this level was reached with 2–3 en% linoleic acid. The 22:5n-6 level doubled as the linoleic acid intake rose from 0.4 to 7.1 en%. With 22:6n-3, the reverse was observed. A significant incorporation of 20:3n-9, an indicator of essential fatty acid deficiency, could not be detected, not even at the lowest intake of linoleic acid (0.4 en%).

The effects of dietary *trans* fatty acids on the fatty acid composition of PC were more pronounced than those of linoleic acid in the range 0.4–7.1 en%. Compared to the two reference diets (groups 7 and 8), the diets containing *trans* fatty acids decreased the amount of saturated fatty acids (mainly 18:0) irrespective of the linoleic acid level. The 18:1 levels were doubled, probably because of the incorporation of *trans*-18:1 (in this experiment, no differentiation was made between *cis* and *trans* fatty acids). The 18:2 level had increased significantly, which can be explained only partially by the incorporation of the *trans* isomers of linoleic acid. See below.

The arachidonic acid level of PC in the reference groups is only slightly higher than that in the corresponding *trans* fatty acid groups. The fatty acids, 22:5n-6 and 22:6n-3, however, were more affected by the change in dietary fatty acids. The amount of total n-6 fatty acids in the groups containing *trans* fatty acid is similar to that of the corresponding reference group, despite all changes in the levels of n-6 fatty acids (including 18:2).

In the second experiment, heart mitochondrial PC contained 6.0% *trans* 18:1 in the group fed PHSO (about 40% dietary monoenoic *trans* fatty acids) (Table 10). A decrease of 50% in dietary *trans* fatty acids (PHSOMix) more than halved the *trans* 18:1 content. The incorporation of *trans* isomers of linoleic acid was low: maximally 0.5% of *trans,trans*-isomers and 0.6% *trans,cis* or *cis,trans* isomers in PC.

TABLE 8

Phospholipid Composition (%) of Rat Heart Mitochondria, Second Experiment[a]

Group	Phospholipid					
	PC[b]	PE[c]	PI[d]	PS[e]	CL[f]	S[g]
CB	36.0 ± 1.2	—	—	—	—	—
OV	36.8 ± 1.0	33.2 ± 0.2	4.8 ± 0.2	2.1 ± 0.8	20.0 ± 0.5	3.0 ± 0.4
PHSO	35.2 ± 0.4	30.6 ± 1.6	5.2 ± 0.6	2.1 ± 0.5	23.5 ± 0.4	3.3 ± 0.8
PHSOMix	34.9 ± 0.8	35.0 ± 0.9	5.7 ± 0.5	2.4 ± 0.6	19.4 ± 0.7	2.5 ± 0.5

[a]Each value represents the mean value ± s.e.m. of three pools of four animals.
[b]PC, phosphatidylcholine.
[c]PE, phosphatidylethanolamine.
[d]PI, phosphatidylinositol.
[e]PS, phosphatidylserine.
[f]CL, cardiolipin.
[g]S, sphingomyelin.
—, not determined.

TABLE 9

Fatty Acid Composition (%) of Phosphatidylcholine from Rat Heart and Liver Mitochondria, First Experiment[a]

Organ	Type of fatty acid[b]	Groups							
		1	2	3[c]	4	5	6	7[d]	8[d]
Heart	16:0	10.6	10.8	10.5	10.2	10.8	11.0	12.4	12.3
	16:1	1.2	2.5	1.2	1.1	1.1	1.4	0.6	0.6
	18:0	17.0	17.0	17.3	17.9	17.2	16.5	28.6	26.6
	18:1[e]	25.6	23.4	22.3	21.6	21.2	20.7	10.2	11.3
	18:2[e]	9.6	7.6	7.9	8.2	9.2	9.0	3.7	3.1
	20:4 n-6	26.8	29.4	34.0	34.9	33.1	33.5	35.6	36.4
	22:5 n-6	1.0	1.0	1.2	1.4	1.8	2.1	2.1	2.2
	22:6 n-3	2.6	3.1	1.8	1.5	1.5	1.3	3.0	3.4
	sum	94.4	94.8	96.2	96.8	95.9	95.5	96.2	95.9
	total n-6[f]	37.4	38.0	43.1	44.5	44.1	44.6	41.4	41.7
	total sat[f]	27.6	27.8	27.8	28.1	28.0	27.5	41.0	38.9
Liver	16:0	12.7	12.5	11.7	10.0	10.4	10.2	14.8	16.0
	16:1	2.4	1.9	2.5	2.1	1.5	1.6	1.4	1.2
	18:0	18.0	16.9	15.7	17.8	15.3	15.5	26.2	22.8
	18:1[e]	25.1	23.8	22.6	21.5	22.2	21.6	9.9	11.4
	18:2[e]	9.1	9.6	9.5	8.2	9.7	9.2	3.8	3.1
	20:3 n-9	4.0	2.8	1.0	0.8	—	—	0.9	—
	20:3 n-6	1.6	1.6	1.1	0.8	0.8	—	0.3	—
	20:4 n-6	16.8	19.5	25.9	29.7	32.6	33.5	30.5	33.3
	22:5 n-6	3.2	4.1	3.5	2.7	2.7	3.1	3.1	2.6
	22:6 n-3	4.2	4.0	2.5	1.9	1.6	1.4	3.6	4.3
	sum	97.1	96.7	96.0	95.5	96.7	96.1	94.5	94.7
	total n-6[f]	30.7	34.8	40.0	41.4	45.7	45.8	37.7	39.0
	total sat[f]	30.7	29.4	27.4	27.8	25.7	25.7	41.0	38.8

[a]Each value represents the mean of two pools of six animals.

[b]The notation indicates chain length and number of double bonds.

[c]Mean of four pools of six animals.

[d]References.

[e]Both *cis* and *trans*-isomers.

[f]Total n-6 and total sat = the sum of the relative amounts of n-6 polyunsaturated fatty acids and 18:2, and saturated fatty acids, respectively.

The diets containing *trans* fatty acids (PHSO and PHSOMix) increased the 18:1-level and decreased the 18:0 level in PC compared to the other diets. The amount of total saturated fatty acids and *trans*-18:1 together is fairly constant over all groups, except for the CB group, in which this sum is considerably higher. The 18:2 level in the groups fed *trans* fatty acids was significantly higher than that in the other two groups (18:2 levels in the pooled samples ranged from 7.5–8.0 in the PHSO group, and from 3.0–3.6 in the OV group), despite the same content of linoleic acid in the diets. The level of 9-*cis*,12-*cis*-18:2 (linoleic acid) as found by capillary GLC clearly was lower than that found in the fatty acid analyses by packed-column GLC. This difference could not be ascribed to *trans* isomers of linoleic acid because of the low level of these components in the phospholipids investigated. *Cis,cis* isomers of linoleic acid were only present in trace amounts. So, since both capillary GLC and packed-column GLC indicated the difference in the linoleic acid level of PC in the PHSO and PHSOMix groups (20 and 10 en% *trans* fatty acids, respectively), it must be concluded that *trans* fatty acids increased the level of linoleic acid in this phospholipid. The arachidonic acid level in the two groups fed *trans* fatty acids diminished compared to that of the other two groups (levels in the PHSO group ranging from 22.2–26.2, in the OV group from 27.2–29.1). However, there is no apparent relation with the amount of dietary *trans* fatty acids.

Liver. Liver mitochondrial PC (Table 9) has a fatty acid pattern similar to that of heart mitochondrial PC. Mitochondrial PC from the liver was more susceptible to changes in linoleic acid intake than that from the heart. In PC, 20:3n-9 was present at low levels of linoleic acid but not at higher levels.

Trans fatty acids decreased the arachidonic acid level in liver mitochondrial PC only at the linoleic acid levels of 2 en%; at the 5 en% level, this effect had disappeared. The 18:2 level increased, irrespective of its dietary level. *Trans* fatty acids had no effect on the level of 20:3n-9.

Fatty acid composition of phosphatidylethanolamine: heart. The effects of increasing amounts of dietary linoleic acid (first experiment) on both saturated and monounsaturated fatty acids, and 18:2 content of heart mitochondrial PE were similar to those in heart mitochondiral PC

TABLE 10

Fatty Acid Compositions (%) of Phosphatidylcholine from Rat Heart Mitochondria as Found by Packed Column GLC (A) and by Capillary Column GLC (B), Second Experiment[a]

	Type of fatty acid[b]	Group CB	OV	PHSO	PHSOMix
A	14:0	0.1	0.3	0.2	0.3
	15:0	2.2	2.2	4.0	2.6
	15:1	0.5	0.3	0.4	0.5
	16:0	13.0	14.0	13.3	14.4
	16:1	0.3	0.5	1.0	0.7
	18:0	39.8	30.3	19.8	30.8
	18:1[c]	3.2	10.9	21.4	13.0
	18:2[c]	3.8	3.3	7.9	4.0
	20:4 n-6	30.6	28.2	24.0	23.8
	20:1 n-9	0.2	0.5	0.2	0.3
	20:2 n-9	—	—	0.1	—
	22:1 n-9	0.4	0.1	0.1	0.4
	22:5 n-6	2.2	2.2	2.6	3.6
	22:6 n-3	1.7	2.7	1.2	1.8
	unknown	1.2	1.3	1.3	1.7
	sum	98.2	96.8	97.5	97.9
	total n-6[d]	35.6	33.7	34.5	31.4
	total sat[d]	55.1	46.8	37.3	48.1
B	18:1 *t*			6.0	2.5
	18:1 *c*			9.6	7.5
	18:1 total			15.6	10.0
	18:2 *t,t*			0.5	—
	18:2 *c,t, t,c*			0.6	0.5
	18:2 *c,c*			4.4	2.5
	18:2 total			5.5	3.0

[a]Each value represents the mean value of four pools of six animals.
[b]The notation indicates chain length and number of double bonds; *c* = *cis*-isomer, *t* = *trans*-isomer.
[c]Both *cis* and *trans*-isomers.
[d]Total n-6 and total sat = the sum of the relative amount of n-6 fatty acids and 18:2 and saturated fatty acids, respectively.

TABLE 11

Fatty Acid Composition (%) of Phosphatidylethanolamine from Rat Heart and Liver Mitochondria, First Experiment[a]

Organ	Type of fatty acid[b]	Group 1	2	3[c]	4	5	6	7[d]	8[d]
Heart	16:0	4.4	5.4	4.9	4.3	4.6	4.7	7.6	7.0
	16:1	1.2	1.2	1.2	1.0	1.3	1.7	0.8	0.8
	18:0	18.3	18.9	18.5	19.0	18.4	17.9	27.6	25.1
	18:1[e]	19.8	18.5	18.8	18.5	18.1	17.9	8.0	9.8
	18:2[e]	4.9	6.1	5.8	6.2	6.8	6.9	3.1	2.9
	20:4 n-6	29.9	29.8	30.2	30.7	28.2	26.7	22.9	21.6
	22:5 n-6	4.5	4.7	6.6	7.4	8.9	10.7	10.6	10.5
	22:6 n-3	10.2	9.4	7.8	6.3	6.0	5.4	12.4	14.0
	sum	93.2	94.0	93.8	93.4	92.3	91.9	93.0	91.7
	total n-6[f]	39.3	40.6	42.6	44.3	43.9	44.3	36.6	35.0
	total sat[f]	22.7	24.3	23.4	23.3	23.0	22.6	35.2	32.1
Liver	16:0	10.1	10.0	8.6	9.8	7.4	9.2	12.5	12.2
	16:1	2.5	2.2	1.6	2.1	1.5	1.7	1.4	0.9
	18:0	16.8	16.3	16.4	15.6	15.4	14.1	25.2	21.6
	18:1[e]	19.5	19.1	19.3	19.5	19.9	17.7	9.4	12.2
	18:2[e]	10.3	9.3	11.2	14.6	14.5	12.7	5.5	5.4
	20:3 n-9	2.2	1.5	0.7	0.5	0.4	0.2	0.6	0.3
	20:3 n-6	1.2	1.1	1.0	0.8	0.7	0.6	0.7	0.4
	20:4 n-6	21.1	22.4	27.1	27.3	28.8	25.9	28.0	28.8
	22:5 n-6	4.1	5.7	4.8	4.3	4.1	4.9	4.5	4.5
	22:6 n-3	8.2	7.9	4.8	3.7	2.9	2.8	7.4	8.5
	sum	96.0	95.5	95.5	98.2	95.6	89.8	95.2	94.8
	total n-6[f]	36.7	38.5	44.1	47.0	48.1	44.1	38.7	39.1
	total sat[f]	26.9	26.3	25.0	25.4	22.8	23.3	37.7	33.8

[a]Each value represents the mean of two pools of six animals.
[b]The notation indicates chain length and number of double bonds.
[c]Mean of four pools of six animals.
[d]References.
[e]Both *cis* and *trans*-isomers.
[f]Total n-6 and total sat = the sum of the relative amounts of n-6 polyunsaturated fatty acids and 18:2, and saturated fatty acids, respectively.

(Table 11). However, in this phospholipid the arachidonic acid level was much less affected by an increasing linoleic acid intake than it was in PC; it even tended to decrease slightly as the dietary level of its precursor increased. The effects of dietary *trans* fatty acids on the fatty acid composition of PE and PC did not differ very much either except for a striking increase in arachidonic acid levels in PE caused by *trans* fatty acids. This increase was compensated for by a reduction in 22:5n-6 and 22:6n-3 fatty acids.

In the second experiment (Table 12), similar changes occurred. *Trans* fatty acids caused a significant increase in the sum of the n-6 polyunsaturated fatty acids; these fatty acids were incorporated partially at the expense of 22:6n-3. Unfortunately, determination of the fatty acid composition of PE of the CB group was impossible. In PE, *trans* monoenoic and dienoic acids were incorporated to an extent similar to that in PC (*trans*-18:1 max. 7.1%; *trans*-18:2 isomers max. 0.9%). The sum of all saturated fatty acids and *trans* 18:1 in the PHSO group was slightly lower than that in the other two groups.

Liver. The influence of dietary linoleic acid on the fatty acid composition of PE in the liver mitochondria (Table 11) was similar to that of PC (Table 9) with respect to the levels of arachidonic acid, 22:5n-6 and 22:6n-3. There was far less similarity in this respect between PE from liver on the one hand and heart on the other. Compared to the reference groups, the 22:6n-3 level was significantly lower in the *trans* fatty acid groups. Moreover, it decreased with an increasing level of linoleic acid. Arachidonic acid was not influenced by *trans* fatty acids.

DISCUSSION

We observed that *trans* fatty acids could significantly alter the fatty acid composition of mitochondrial membranes, irrespective of the linoleic acid level. However, with 2 and 5 en% linoleic acid in the diet, no influence of *trans* fatty acids on the function of the mitochondria was found. Also, in the second experiment no differences in mitochondrial function were detected between the two groups that were fed *trans* fatty acids and the two reference groups.

TABLE 12

Fatty Acid Composition (%) of Phosphatidylethanolamine from Rat Heart Mitochondria as Found by Packed Column GLC (A), and by Capillary Column GLC (B), Second Experiment[a]

	Type of fatty acid[b]	Group		
		OV	PHSO	PHSOMix
A	14:0	0.3	0.3	0.3
	15:0	2.3	2.9	2.1
	15:1	1.3	0.7	1.0
	16:0	9.7	6.8	8.1
	16:1	0.2	0.4	0.8
	18:0	29.1	19.7	26.7
	18:1	10.3	19.1	12.3
	18:2	3.1	5.9	3.5
	20:4 n-6	18.3	23.5	21.1
	20:1 n-9	1.1	0.4	0.2
	20:2 n-9	0.2	0.2	0.7
	22:1 n-9	0.4	—	0.1
	22:5 n-6	4.7	7.6	7.2
	22:6 n-3	13.3	6.7	9.1
	unknown	1.5	1.3	1.8
	sum	95.8	95.4	94.9
	total n-6[c]	26.1	37.0	31.8
	total sat[c]	41.4	29.6	37.1
B	18:1 *t*		7.1	2.7
	18:1 *c*		6.9	6.5
	18:1 total		14.0	9.2
	18:2 *t,t*		0.2	0.2
	18:2 *ct, tc*		0.7	0.5
	18:2 *c,c*		3.6	2.3
	18:2 total		4.5	3.7

[a]Each value represents the mean value of four pools of six animals.
[b]The notation used for the fatty acids indicates chain length and number of double bonds, *c* = *cis*-isomer, *t* = *trans*-isomer.
[c]Total n-6 and total sat = the sum of the relative amounts of n-6 fatty acids and 18:2 and saturated fatty acids, respectively.

The effect of *trans* fatty acids on the mitochondrial respiration has been the subject of many publications (29–34). It generally is agreed that specific effects of *trans* fatty acids on the mitochondrial respiration cannot be detected, provided an adequate level of linoleic acid is present in the diet (31,32,34). We have demonstrated that 2 en% linoleic acid is enough to prevent effects of *trans* fatty acids on the mitochondrial respiration.

The high RCR and ADP/O-values in the first experiment show that the mitochondria were coupled tightly. The QO_2 and ATP-values also were high compared with the results of other investigators (30,31,34). In the second experiment, the values for QO_2 (and consequently the ATP synthesis) were much lower than those in the first experiment and even lower than those found by other investigators (30,31,34). However, the RCR and ADP/O values demonstrate the good quality of these mitochondria. This low QO_2 value probably can be ascribed to the use of a different batch of nagarose in the second experiment, which may have resulted in a mitochondrial fraction contaminated with another protein-rich fraction.

Many adverse effects have been attributed to linolelaidic acid (9-*trans*,12-*trans*-18:2) on the basis of both in vitro experiments and feeding studies (36–39). However, as has been pointed out in some reviews the amount of this fatty acid in commercial partially hydrogenated oils is very low (1–3,35). As a consequence, the total dietary intake of 9-*trans*,12-*trans*-18:2 by humans can be neglected.

Monoenoic *trans* fatty acids in partially hydrogenated oils and ruminant fats form a heterogenous group of positional isomers (4,40). The metabolism of the isomers may differ significantly (3); the biological effects of the isomers therefore could vary accordingly. In most experiments dealing with *trans* fatty acids, use is made of PHSO; the advantage is that both monoenoic and polyenoic *trans* fatty acids are found in the experimental diet. Moreover, in PHSO the distribution of these fatty acid isomers matches that of the human diet. The conclusion drawn with regard to the nutritive value of food products containing *trans* fatty acids therefore is more realistic.

As can be concluded from the capillary GC analysis of the fatty acid composition (second experiment), the incorporation of *trans* isomers of linoleic acid is low in heart mitochondrial phospholipids. *Trans* monoenoic fatty acids are incorporated readily in these phospholipids, their level depending on the diet. The changes in the fatty acid composition of the heart mitochondrial phospholipids in both experiments corresponded well except for a slight difference in the relative levels of the main fatty acids probably caused by contamination of the mitochondrial fraction in the isolation of the mitochondria in the second experiment.

The fatty acid composition of heart mitochondrial PC in the HCNO/OV-group (group 7) of the first experiment corresponds well with that of the CB group of the second experiment. The influence of the medium chain saturated fatty acids, as present in the HCNO/OV-diet, seemed to be similar to that of long chain saturated fatty acids. Although the polyunsaturated fatty acid compositions of heart mitochondrial phospholipids were affected considerably by a decrease of the linoleic acid intake, even at very low linoleic acid levels (0.4 en%) no 20:3n-9 appeared. This is in contrast to the liver phospholipids and may indicate a highly specific uptake or incorporation of polyunsaturated fatty acids by the heart.

Trans fatty acids (both 10 and 20 en%) when compared with saturated fatty acids and *cis*-monounsaturated fatty acids, decreased the arachidonic acid level in heart mitochondrial PC. However, in PE the arachidonic acid level increased and partially was compensated for by a decrease in 22:6n-3. These may be effects of dietary *trans* fatty acids on PE in heart tissue (30,31,41). The regulation of polyunsaturated fatty acids of heart mitochondrial PC probably is different from that of PE (31).

In liver mitochondrial PC, the *trans* fatty acids decreased the arachidonic acid level only at a linoleic acid level of 2 en% or lower. The arachidonic acid content of PE in liver mitochondria did not change upon the incorporation of *trans* fatty acids in the diet. The linoleic acid level, however, had increased upon incorporation of *trans* fatty acids in both PE and PC.

The effect of the *trans* fatty acids in PHSO on the fatty acid composition of PC is different from that on PE. The mechanism responsible for the effects is not clear yet. However, *trans* fatty acids change the fatty acid composition in both phospholipids, compared to saturated or

cis-monounsaturated fatty acids. Despite these changes, the C18 *trans* fatty acids do not influence the mitochondrial function in the presence of 2 en% or more linoleic acid. This supports the view of Royce and Holmes (34) that, in non-EFA-deficient animals, the mitochondrial membranes adapt to a changing fatty acid composition in a way that leaves their functional properties unaffected.

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REFERENCES

1. Beare-Rogers, J.L. (1983) in *Advances in Nutritional Research* (Draper, H.H., ed.) Vol. 5, pp. 171-200, Plenum Press, New York, NY.
2. Gurr, M.I. (1983) *Bull. Int. Dairy Fed. Doc. 166,* 5-18.
3. Emken, E.A. (1983) *J. Am. Oil Chem. Soc. 60,* 995-1004.
4. Ohlrogge, J.B. (1983) in *Dietary Fats and Health* (Perkins, E.G., and Visek, W.J., eds.) pp. 359-374, American Oil Chemists' Society, Champaign, IL.
5. Wood, R. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.) pp. 213-281, American Oil Chemists' Society, Champaign, IL.
6. Gottenbos, J.J. (1983) in *Dietary Fats and Health* (Perkins, E.G., and Visek, W.J., eds.) pp. 375-390, American Oil Chemists' Society, Champaign, IL.
7. Stancliff, R.C., Williams, M.A., Utsumi, K., and Packer, L. (1969) *Arch. Biochem. Biophys. 131,* 629-642.
8. Divakaran, P., and Venkataraman, A. (1977) *J. Nutr. 107,* 1621-1631.
9. Clandinin, M.T. (1978) *J. Nutr. 108,* 273-281.
10. Divakaran, P. (1978) *Experientia 34,* 1540-1541.
11. Clandinin, M.T. (1979) *FEBS Lett. 102,* 173-176.
12. Abuirmeileh, N.M., and Elson, C.E. (1980) *Lipids 15,* 918-924.
13. Tyzbir, R.S., Kunin, A.S., Sims, N.M., and Danforth, E. Jr. (1981) *J. Nutr. 111,* 252-259.
14. McMurchie, E.J., Abeywardena, M.Y., Charnock, J.S., and Gibson, R.A. (1983) *Biochim. Biophys. Acta 734,* 114-124.
15. Houtsmuller, U.M.T., Stwijk, C.B., and Van der Beek, A. (1970) *Biochim. Biophys. Acta 218,* 564-566.
16. Renner, R., Innis, S.M., and Clandinin, M.T. (1979) *J. Nutr. 109,* 378-387.
17. Høy, C.E., and Hølmer, G. (1979) *Lipids 14,* 727-733.
18. Applewhite, T.H. (1983) in *Dietary Fats and Health* (Perkins, E.G., and Visek, W.J., eds.) pp. 414-424, American Oil Chemists' Society, Champaign, IL.
19. De Deckere, E.A.M., Jansen-Verplanke, C., Blonk, C.G., and Van Nielen, W.G.L. (1988) *J. Nutr. 118,* 11-16.
20. Scholfield, C.R. (1981) *J. Am. Oil Chem. Soc. 58,* 662-663.
21. Clouet, P., and Bezard, J. (1979) *Lipids 14,* 268-273.
22. Hülsmann, W.C. (1970) *Biochem. J. 166,* 32-33.
23. Swarttouw, M.A. (1974) *Biochim. Biophys. Acta 337,* 13-21.
24. Estabrook, R.W. (1967) in *Methods in Enzymology* (Estabrook, R.W., and Pullman, M.E., eds.) Vol. 10, pp. 41-47, Academic Press, New York and London.
25. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
26. Patton, G.M., Fasulo, J.M., and Robins, S.J. (1982) *J. Lipid Res. 23,* 190-196.
27. Christie, W.W. (1973) in *Lipid Analysis* (Christie, W.W., ed.) p. 191, Pergamon Press, Oxford, England.
28. Bartlett, G.R. (1959) *J. Biol. Chem. 234,* 466-470.
29. Hsu, C.M.L., and Kummerow, F.A. (1977) *Lipids 12,* 486-494.
30. Combe, N., Rietsch, J., Wolff, R., and Entressangles, B. (1980) *Ann. Nutr. Aliment. 34,* 305-316.
31. Blomstrand, R., and Svensson, L. (1983) *Lipids 18,* 151-170.
32. Entressangles, B., Combe, N., and Wolff, R. (1984) *J. Am. Oil Chem. Soc. 61,* 679.
33. De Schrijver, R., and Privett, O.S. (1984) *J. Nutr. 114,* 1183-1191.
34. Royce, S.M., and Holmes, R.P. (1984) *Biochim. Biophys. Acta 792,* 371-375.
35. Houtsmuller, U.M.T. (1978) *Fette Seifen Anstrichm. 80,* 162-169.
36. Privett, O.S., Phillips, F., Shimasaki, H., Nozawa, T., and Nickell, E.C. (1977) *Am. J. Clin. Nutr. 30,* 1009-1017.
37. Kinsella, J.E., Bruckner, G., Mai, J., and Shimp, J. (1981) *Am. J. Clin. Nutr. 34,* 2307-2318.
38. Srivastava, K.C., and Awasthi, K.K. (1982) *Prostagland. Leuk. Med. 9,* 669-684.
39. Goswami, S.K., Bruckner, G., and Kinsella, J.E. (1983) *Prostagland. Leuk. Med. 10,* 19-26.
40. Dutton, H.J. (1979) in *Geometric and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, J.H., eds.) pp. 1-6, American Oil Chemists' Society, Champaign, IL.
41. Wolff, R.L., Combe, N., and Entressangles, B. (1984) *Rev. Fr. Corps Gras 11,* 161-170.

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Hypocholesterolemic Action of Chitosans with Different Viscosity in Rats

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The relationship between hypocholesterolemic efficacy and average molecular weight of chitosan was studied in rats fed a cholesterol-enriched (0.5%) diet. Several chitosan preparations with a comparable degree of deacetylation but differing widely in average molecular weight, as demonstrated by viscosity, almost completely prevented the rise of serum cholesterol at the 5% dietary level. At the 2% level, chitosans with viscosities at both extremes exerted a comparable cholesterol-lowering action. The glucosamine oligomer composed mainly of three to five aminosugar residues was not effective. The results indicate that the hypocholesterolemic action of chitosans is independent of their molecular weight within the tested viscosity range.

Lipids 23, 187–191 (1988).

Chitosan, a polymer of glucosamine, has been shown to have a potent hypocholesterolemic effect in rats (1–6). Although the mechanism of the cholesterol-lowering action of chitosan is not well understood, the aminosugar polymer appears to interact with bile acid and/or cholesterol in the intestinal lumen and to stimulate fecal excretion of neutral steroids (1,3) by interfering with the absorption process (7,8). Since the viscosity could be responsible for the effective hypocholesterolemic potential of dietary fiber and since the intra-luminal behavior may differ with respect to the viscosity (9,10), it seems worthwhile to know the relationship between the viscosity (hence, the molecular weight) and the cholesterol-lowering effect of chitosan. Although the viscosity of chitosan preparations commercially available differs widely (10), previous studies (1–5) have not examined the influence of the viscosity, except for one preliminary study (6).

In view of these considerations, the effect of the difference in the viscosity of chitosan on the serum cholesterol level of rats was investigated. The efficacy of the oligomer of glucosamine with water-like viscosity also was tested.

MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats purchased from Seiwa Experimental Animals (Fukuoka, Japan) were housed individually in an air-conditioned room (20–23 C, lights on 0800–2000 hr) and acclimated for three to four days before starting the experiments. Three sets of experiments were performed. In experiment I with five-wk old rats, five groups with eight animals in each group were fed experimental diets ad libitum for 21 days to examine the effect of the difference in the viscosity of chitosan on the serum cholesterol level. In experiment II with four-wk old rats, six groups with six to seven animals in each group were fed for 22 days to examine the effect of the oligomer of glucosamine as well as chitosans with different viscosities on the plasma cholesterol levels. In experiment III with four-wk old rats, five groups with six animals in each group were fed for 28 days to determine the effect of the glucosamine oligomer. We previously have shown that these age differences of animals at the start of experiments and lengths of feeding did not alter the observed effects on serum cholesterol (1,4). The composition of the diet was, in weight percent: casein, 20; lard, 10; vitamin mixture, 1; mineral mixture, 4; choline bitartrate, 0.2; cholesterol, 0.5; Na-cholate, 0.125; test materials, 5 (Exp. I), 4 (Exp. III, one group of rats received a mixture of 2%-oligomer and 2% pectin), or 2 (Exp. II); and sucrose, to 100. Vitamin and mineral mixtures according to Harper (11) were purchased from Oriental Yeast Co. (Tokyo). In all experiments, after depriving the rats of diets for five hr (0830–1330 hr), blood was withdrawn weekly from the tail vein for enzymatic measurement of serum cholesterol (Cholesterol C-Test, Wako Pure Chemicals, Osaka). Finally, animals were killed by decapitation after overnight fasting (1800–0900 hr), blood was collected, and the liver and cecum were excised.

Materials. Samples of chitosan, prepared from crab shell chitin by alkali fusion, were provided by Katakura Chikkarin, Co. (Tokyo), and their properties are listed in Table 1. The glucosamine oligomer, prepared by hydrolyzing chitosan with microbial chitosanase (12), was used as a lactate salt. The preparation was composed of di- to heptamers with tri- to pentamers as major constituents. Two kinds of lignins, containing sodium lignosulfonate as a major component (60%, SAN X-P252, Lignin A) or purified and partially desulfonated sodium ligninosulfonate (97%, Vanillex Rn, Lignin B), were a gift from the Sanyo-Kokusaku Pulp Co. (Tokyo). Pectin (citrus) was purchased from Nakarai Chemicals (Kyoto).

Lipid analyses. Serum and liver cholesterol (13), triglyceride (14) and phospholipid (15) were measured as reported.

Statistical analysis. The results were analyzed by a one-way analysis of variance followed by the inspection of all differences between pairs of means by Student's t-test at $p < 0.05$ (16).

RESULTS

Food intake, growth and liver weight. The results of food intake, growth and liver weight are summarized in Table 2. In rats fed diets containing chitosan at the 5% level (Exp. I), weight gain was significantly lower than that in rats fed cellulose, except for group two, although food intake was similar. However, at the dietary level of 2% or 4%, chitosan reduced neither body weight gain nor food intake (Exps. II and III). The glucosamine oligomer at the 4% but not at the 2% level caused a significant reduction of food intake and, hence, weight gain (Exp. III). However, when the oligomer was fed at the 2% level in combination with the same amount of pectin, it did not

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TABLE 1

Properties of Chitosan and Glucosamine Oligomer

Properties	Experiment I				Experiment II			Experiment III	
	Chitosan				Chitosan		Glucosamine oligomer 1	Chitosan	Glucosamine oligomer 2
	1	2	3	4	5	6		7	
Moisture (%)	4.20	3.10	6.74	5.60	3.66	4.82	15.7	4.68	16.3
Ash (%)	0.20	0.40	0.07	0.08	0.05	0.06	0.51	0.04	1.11
Viscosity (cps)[a]	1620	525	200	17	1450	30	1.1	1350	1.1
Deacetylation (%)	81	94	88	79	86	99	99	87	99

[a]Viscosity was measured on 0.5% solution of dried materials in 0.5% acetic acid at 20 C by the rotory viscosimeter (Tokyo Keiki Seisakusho, Type B).

TABLE 2

Weight Gain, Food Intake and Liver Weight

Groups	Weight gain (g)	Food intake (g/day)	Liver (g/100 g body weight)	Cecum (g/100 g body weight)
Experiment I				
Cellulose	145 ± 5[a]	19.8 ± 0.5	4.70 ± 0.08[a]	0.33 ± 0.04[a,b]
Chitosan 1	104 ± 8[b]	19.0 ± 0.9	3.79 ± 0.20[b]	0.35 ± 0.02[a,b]
Chitosan 2	123 ± 7[a,b]	20.6 ± 0.6	3.67 ± 0.08[b]	0.31 ± 0.03[a]
Chitosan 3	103 ± 12[b]	19.0 ± 1.1	3.55 ± 0.13[b]	0.40 ± 0.04[b,c]
Chitosan 4	106 ± 10[b]	17.9 ± 0.7	3.57 ± 0.07[b]	0.46 ± 0.02[c]
Experiment II				
Cellulose	144 ± 6	17.1 ± 0.5	5.11 ± 0.14[a]	—
Chitosan 5	135 ± 6	17.8 ± 0.5	4.33 ± 0.20[b]	—
Chitosan 6	148 ± 7	18.0 ± 0.6	4.48 ± 0.16[b]	—
Glucosamine oligomer 1	141 ± 5	16.3 ± 0.2	4.94 ± 0.08[a]	—
Lignin A	131 ± 9	16.7 ± 0.5	5.11 ± 0.07[a]	—
Lignin B	149 ± 5	16.6 ± 0.3	5.13 ± 0.11[a]	—
Experiment III				
Cellulose	218 ± 12[a]	19.3 ± 0.7[a]	4.98 ± 0.09[a]	—
Chitosan 7	189 ± 5[a]	19.4 ± 0.4[a]	3.02 ± 0.54[b]	—
Glucosamine oligomer 2	151 ± 12[b]	14.8 ± 0.8[b]	4.55 ± 0.11[a]	—
Gulusosamine oligomer 2 + pectin (1:1)	183 ± 15[c]	16.8 ± 0.7[c]	4.62 ± 0.06[a]	—
Pectin	184 ± 6[c]	17.2 ± 0.4[c]	4.48 ± 0.08[a]	—

Weight gain for 21, 22 and 28 days in Experiments I, II and III, respectively. Average initial body weight was 134, 100 and 100 g, and supplementary levels of test materials were 5, 2 and 4% in Experiments I, II and III, respectively.

[a,b,c]Values are expressed as mean ± SE of eight, six to seven and five rats for Experiments I, II and III, respectively, and in each experiment those in the same column not sharing a common superscript are significantly different at $p < 0.05$.

cause a reduction in weight gain. The relative liver weight was significantly lower in all chitosan groups than in corresponding cellulose groups, while oligomer and pectin were without effect. The weight of the cecum tended to be heavier in rats fed low viscosity chitosans. However, the weight of the cecal content was comparable among the different groups (mean values, 1.02–1.18 g).

Time course of serum cholesterol levels. Figure 1 shows that chitosan at the 5% level was extremely effective in preventing the rise of serum cholesterol due to feeding cholesterol (Exp. 1). The levels attained were almost similar to those of rats given a cholesterol-free diet (the starting level), and no difference could be seen in the hypocholesterolemic effect among chitosan preparations with various viscosities.

As Figure 2 shows, the hypocholesterolemic effect of chitosan still was marked even at the 2% dietary level as compared to that of cellulose (Exp. II). Although the cholesterol-lowering activity appeared to be somewhat greater in chitosan with a high viscosity, the difference was not significant statistically. Also, the glucosamine oligomer was not effective in lowering serum cholesterol, and the levels apparently were similar to those observed in the cellulose group. Two lignin preparations induced

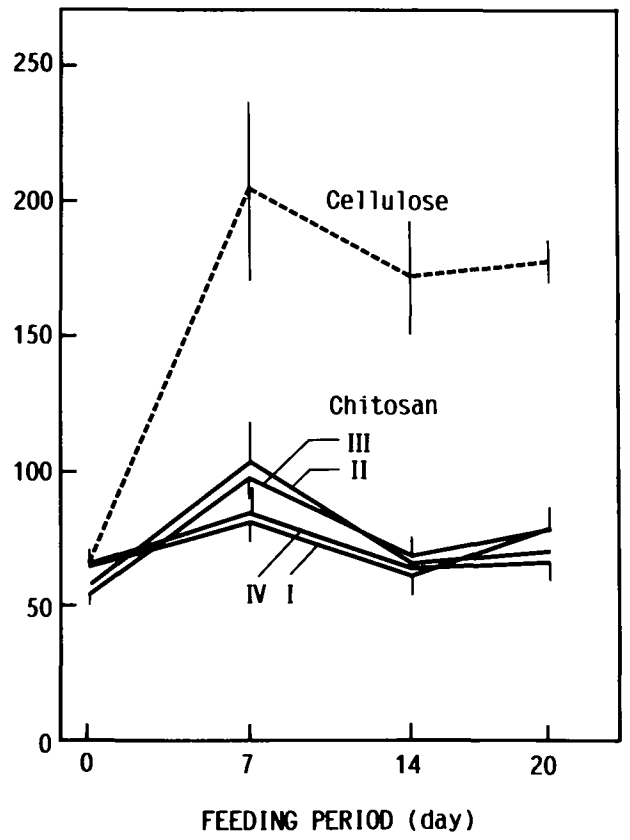

FIG. 1. Time course of the effects of chitosan with various viscosities on serum cholesterol levels (Experiment I). Each point represents mean and SE of eight rats. Cholesterol levels in rats fed chitosan are all significantly different ($p < 0.05$) from the corresponding values for rats fed cellulose.

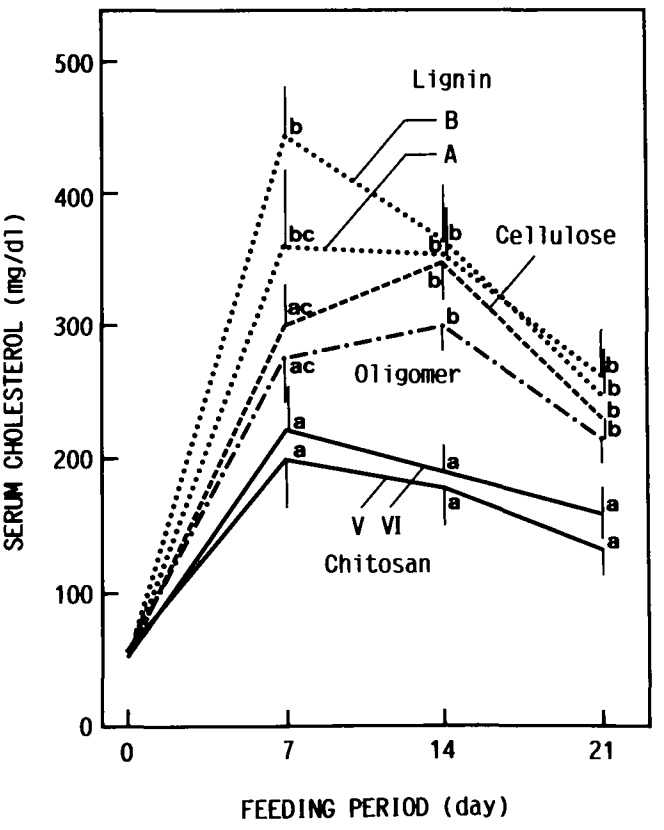

FIG. 2. Time course of the effects of chitosan and glucosamine oligomer on serum cholesterol levels (Experiment II). Each point represents mean and SE of six to seven rats. Values in the same time point not sharing a common letter are significantly different at $p < 0.05$.

a sharp rise of serum cholesterol above that observed with cellulose in the first week and the levels remained high thereafter.

As shown in Figure 3, chitosan showed a marked hypocholesterolemic effect at the 4% level (Exp. III). The glucosamine oligomer was again not effective in lowering serum cholesterol even when the dietary level was increased to 4% and, in addition, did not strengthen the hypocholesterolemic effect of pectin.

Serum and liver lipid concentrations. In three sets of experiments, there were significant reductions of serum cholesterol after overnight fasting as compared with the levels of the weekly blood samples obtained with five-hr fasting (in 25–45% of the weekly samples). The extent of this reduction was greater in the groups with higher cholesterol levels and, consequently, the variation in cholesterol levels decreased. Thus, the fluctuations seen in Figures 1 to 3 may be due, in part, to the blood sampling five hr after food deprivation. However, the diet-dependent difference still remained in the samples from overnight-fasted rats and the fasting serum cholesterol levels of the chitosan groups were as low as those observed in rats not fed dietary cholesterol (the starting level) in all experiments. The serum triglyceride levels were variable, and no significant differences among the various groups were ever observed in this study. Serum phospholipid concentrations were comparable in all different groups throughout this study.

The results of liver lipid analyses are summarized in Table 3. Liver cholesterol concentrations were decreased markedly in all chitosan groups. Pectin moderately lowered liver cholesterol while the glucosamine oligomer was ineffective. No significant differences were found in concentrations of liver triglyceride among various groups. Liver phospholipid was elevated in five of the seven chitosan groups.

DISCUSSION

This study examined the effect of differences in the viscosity of chitosan preparations on the serum cholesterol levels of rats fed a cholesterol-enriched diet. Since the degree of polymerization of the glucosamine unit is the major determinant of viscosity when the extent of deacetylation is comparable (10), the results can be attributed to the differences in chitosan molecular weight. In Experiment I (Table 1), in which chitosans with various viscosities were added to the diet at the 5% level, the hypocholesterolemic effect relative to cellulose was too marked to differentiate their relative potencies. The marked reductions of liver cholesterol in these rats (Table 3) indicate that chitosan effectively interferes with cholesterol absorption. These observations agreed with those reported previously for specific chitosan preparations (1–5).

When chitosans with viscosity of both extremes were added at the 2% level (experiment II, Table 1), we again

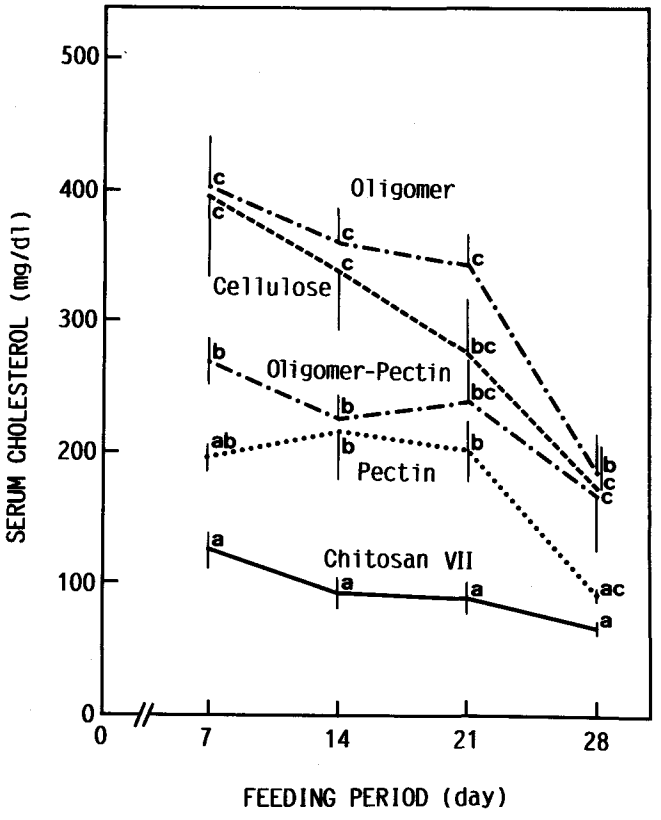

FIG. 3. Time course of the effects of chitosan, glucosamine oligomer and pectin on serum cholesterol levels (Experiment III). Each point represents mean and SE of six rats. Values in the same time point not sharing a common letter are significantly different at $p < 0.05$.

TABLE 3

Liver Lipid Levels (Experiments I to III)

Experiments and groups	Liver lipids (mg/g)		
	Cholesterol	Triglyceride	Phospholipid
Experiment I			
Cellulose	68.9 ± 3.5^a	41.0 ± 4.0	32.9 ± 1.4^a
Chitosan 1	15.8 ± 4.3^b	63.7 ± 17.3	37.6 ± 2.1^b
Chitosan 2	18.3 ± 5.4^b	59.0 ± 12.4	37.1 ± 1.4^{a,b}
Chitosan 3	11.6 ± 3.4^b	34.0 ± 10.7	42.2 ± 1.0^c
Chitosan 4	15.7 ± 2.7^b	39.3 ± 6.2	39.3 ± 1.6^{b,c}
Experiment II			
Cellulose	70.2 ± 2.7^a	61.7 ± 7.2	29.6 ± 0.5^a
Chitosan 5	32.0 ± 4.2^b	45.2 ± 6.7	34.4 ± 1.7^b
Chitosan 6	38.6 ± 2.8^b	61.9 ± 6.2	32.1 ± 1.3^{a,b}
Glucosamine oligomer 1	68.7 ± 5.3^a	45.0 ± 4.4	28.7 ± 0.6^c
Lignin A	72.5 ± 3.9^a	56.2 ± 6.7	28.0 ± 1.2^c
Lignin B	79.0 ± 1.9^a	58.9 ± 5.6	27.0 ± 0.5^c
Experiment III			
Cellulose	43.7 ± 4.3^{a,b}	47.6 ± 2.2	39.2 ± 1.4^a
Chitosan 7	13.1 ± 1.8^c	26.4 ± 2.7	46.3 ± 1.5^b
Glucosamine oligomer 2	53.5 ± 5.8^a	34.1 ± 10.4	38.8 ± 0.9^a
Glucosamine oligomer 2 + pectin (1:1)	36.8 ± 3.0^{b,d}	40.8 ± 6.3	36.1 ± 1.2^a
Pectin	31.7 ± 2.1^d	32.0 ± 8.0	36.5 ± 0.7^a

^{a,b,c}Values are mean ± SE of eight, six to seven and six rats per group in Experiments I, II and III, respectively. In each experiment, values in the same column not sharing a common superscript are significantly different at $p < 0.05$.

confirmed a hypocholesterolemic activity of these two preparations. Although highly viscous chitosan in relation to the chitosan of low viscosity showed a somewhat greater efficacy, the difference was not significant. Ishii et al. (6) reported that the serum cholesterol-lowering action of chitosan was greater in highly deacetylated than in moderately deacetylated preparations when the viscosity was comparable. Judging from the ineffectiveness of the glucosamine oligomer with water-like viscosity, it is suggested that some degree of polymerization is required to provoke a cholesterol-lowering activity.

These observations are favorable for the application of chitosan as a hypocholesterolemic agent because the extent of polymerization of the glucosamine residue may differ greatly depending on both the source of chitin and the extent of alkaline treatment (10). Thus, it is evident that various commercially available chitosan preparations generally are applicable for this purpose.

The nondigestibility in the upper gastrointestinal tract, high viscosity, polymeric nature and high water-binding properties, together with low water binding in the lower gastrointestinal tract, are all responsible for the effective hypocholesterolemic potential of dietary fiber (9). Chitosan meets most of these criteria, and it seems reasonable that the preparation with an appropriate viscosity together with the high degree of deacetylation is preferable for this purpose. In addition, chitosan has a highly characteristic property in relation to other dietary plant fibers; it can bind in vitro a variety of anions such as bile acids or free fatty acids at low pH by ionic bonds resulting from its amino group (10). However, it is not clear whether chitosan could interact with bile acids in the intestinal lumen since it increases the excretion of neutral sterols but not acidic steroids (1,3). Thus, this glucosamine polymer may not be hazardous to the morphology and, hence, the functioning of the intestine, as compared to a bile acid sequester resin, cholestyramine (7,17), or to the viscous fiber, pectin (18), although its hypocholesterolemic activity is comparable to that of cholestyramine (1).

Because of these considerations together with its low toxicity (5,19), chitosan seems to be a realistic hypocholesterolemic agent, although its effect on lipase as observed with a related aminosugar compound, neomycin (20), remains unclear. In addition, chitosan may interfere with the absorption of glycerides by the intestine (7) and thereby may exert a hypoglyceridemic effect under certain nutritional conditions.

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REFERENCES

1. Sugano, M., Fujikawa, T., Hiratsuji, Y., and Hasegawa, Y. (1978) *Nutr. Rep. Int. 18*, 531-537.

2. Kobayashi, T., Otsuka, S., and Yugari, Y. (1979) *Nutr. Rep. Int. 19,* 327-334.
3. Nagyvary, J.J., Falk, J.D., Hill, M.L., Schmidt, M.L., Wilkins, A.K., and Bradbury, E.L. (1979) *Nutr. Rep. Int. 20,* 677-684.
4. Sugano, M., Fujikawa, T., Hiratsuji, Y., Nakashima, K., Fukuda, N., and Hasegawa, Y. (1980) *Am. J. Clin. Nutr. 33,* 787-793.
5. Gordon, D.T., and Besch-Williford, C. (1984) in *Chitin, Chitosan, and Related Enzymes* (Zilahis, J.P., ed.) pp. 97-117, Academic Press, Orlando, FL.
6. Ishii, T., Takeuchi, S., and Tsugita, T. (1986) Proceedings of the Annual Meeting of Japenese Society of Nutrition and Food Science, Abstract, p. 112.
7. Nauss, J.L. Thompson, J.L., and Nagyvary, J.J. (1980) *Lipids 18,* 714-719.
8. Vahouny, G.V., Satchi thanandam, S., Cassidy, M.M., Lightfoot, F.B., and Furda, I. (1983) *Am. J. Clin. Nutr. 38,* 278-284.
9. Story, J.A. (1986) in *Dietary Fiber, Basic and Clinical Aspects* (Vahouny, G.V., and Kirtchevsky, D., eds.) pp. 253-264, Plenum Press, New York, NY.
10. Furda, I. (1983) in *Unconventional Sources of Dietary Fiber* (Furda, I., ed.) pp. 105-122, American Chemical Society, Washington, D.C.
11. Harper, A.E. (1959) *J. Nutr. 68,* 405-424.
12. Izume, M., and Ohtakara, A. (1987) *Agric. Biol. Chem. 51,* 1189-1191.
13. Sperry, W.M., and Webb, M. (1950) *J. Chem. 187,* 97-106.
14. Fletcher, M.J. (1968) *Clin. Chim. Acta 22,* 393-397.
15. Rouser, G., Siakotos, A.N., and Fleischer, S. (1966) *Lipids 1,* 85-86.
16. Snedecor, G.W., and Chochran, W.G. (1970) in *Statistical Methods,* 6th ed., pp. 258-298, Iowa State University Press, Ames, IA.
17. Cassidy, M.M., Lightfoot, F.G., Grau, L., Roy, T., Kritchevsky, D., and Vahouny, G.V. (1980) *Dig. Dis. Sci. 25,* 504-512.
18. Cassidy, M.M., Lightfoot, F.G., Grau, L., Story, J., Kritchevsky, D., and Vahouny, G.V. (1981) *Am. J. Clin. Nutr. 34,* 218-228.
19. Arai, K., Kinumaki, T., and Fujita, T. (1968) *Bull. Tokai Reg. Fish Res. Lab. 56,* 89-94.
20. Rogers, A.I., Vloedman, D.A., Bloom, E.C., and Kakser, M.H. (1966) *J. Am. Med. Assoc. 197,* 117-131.

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Human Placenta Gangliosides

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Gangliosides extracted from human placenta were composed of four major components (G1–G4), which were purified by silica beads column chromatography. The structures of these gangliosides were analyzed by exoglycosidase treatments, 400 MHz proton magnetic resonance spectroscopy and gas chromatography-mass spectrometry and were concluded to be as follows:

G-1, NeuAcα2-3Galβ1-4Glcβ1-1ceramide;

G-2, NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1ceramide;

G-3, NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1ceramide;

G-4, NeuAcα2-3Galβ1-4GlcNAcβ1 (6) (3) Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1ceramide.
NeuAcα2-3Galβ1-4GlcNAcβ1

Gangliosides containing more than 10 sugar residues were observed in addition to the above four major components. But ganglio-series gangliosides were undetectable in the placenta.

Lipids 23, 192–198 (1988).

Gangliosides are characteristic components of plasma membranes. They exist in the outer leaflet of the phospholipid bilayer of plasma membrane, and are involved in the recognition of biological signals from outside the cells. The complexity of ganglioside molecules, the organ specificity of their composition and some biological functions have been reported (1–3). The composition of gangliosides alters in association with differentiation of cells, development of animals and also oncogenic transformation (4–6). The placenta is an unique organ from the viewpoints of cell differentiation, the special relationship between mother and embryo, very rapid proliferation of cells, and selective transport of nutrients and proteins. However, the ganglioside composition of this organ has not been reported yet. In this paper, we describe the ganglioside composition of human placenta and the determination of the structures of four major gangliosides. This organ was characterized by the presence of ganglioside species containing lactosamine or lactosamine-repeating structure as the backbone core.

MATERIALS AND METHODS

Human placenta. The isolated human placentas were washed with water to remove red blood cells completely. Mean wet weight of the organ was 342 g.

Extraction and separation of gangliosides. Placentas were homogenized in 1.5 vol of a mixture of chloroform/methanol (2:1, v/v) with a Waring blender for five min, then suspended in 7 vol of the same solvent mixture. After filtration, the residues were suspended in 4 vol of a mixture of isopropanol/hexane/water (55:25:20, v/v/v) to complete the extraction of higher gangliosides.

Each extract was concentrated under reduced pressure in a rotary evaporator, dialyzed against distilled water and then lyophilized. Total lipids were dissolved in a mixture of chloroform/methanol/water (30:60:8, v/v/v) and applied to a DEAE Sephadex A-25 column according to the method of Ledeen et al. (7). Neutral lipid, phospholipid and neutral glycolipids were eluted with the solvent mixture, and total gangliosides were eluted with a mixture of chloroform/methanol/0.8 M NaOAc (30:60:8, v/v/v). Gangliosides eluted from the column were concentrated under reduced pressure in a rotary evaporator, dialyzed against distilled water and lyophilized.

Purification of gangliosides. Ganglioside fraction was dissolved in 10 ml of a solvent mixture of chloroform/methanol/water (83:16:0.5, v/v/v) and applied to an Iatrobeads column (2.0 × 120 cm) equilibrated with the same solvent mixture. Gangliosides were eluted with a linear gradient formed from 400 ml of chloroform/methanol/water (83:16:0.5, v/v/v) in the mixing chamber and 480 ml of chloroform/methanol/water (20:80:5, v/v/v) in the reservoir. Then gangliosides left in the column were eluted with 400 ml of the second solvent mixture. Fractions of 6 ml were collected. An aliquot was taken from each fraction and checked by thin layer chromatography.

Thin layer chromatography. The purity of each ganglioside was determined by high performance thin layer chromatography (HPTLC) with various solvent systems: A, chloroform/methanol/0.2% $CaCl_2$ (60:35:8, v/v/v); B, chloroform/methanol/0.2% $CaCl_2$ (60:40:9, v/v/v); C, chloroform/methanol/0.2% $CaCl_2$ (60:30:6, v/v/v); D, n-propanol/ammonium hydroxide/water (6:1:3, v/v/v). Gangliosides were visualized by spraying the plate with resorcinol/HCl reagent. Gangliosides were determined by densitometric analysis using GM3 as the standard.

Enzymatic degradation of glycolipids. The carbohydrate sequence and anomeric configuration of gangliosides were determined by sequential application of various exoglycosidases. Hydrolysis using β-galactosidase and β-hexosaminidase (purchased from Sigma Chemical Co., St. Louis, MO) from jack bean was performed as described by Li and coworkers (8,9). Hydrolysis of ganglioside with *Clostridium perfringens* neuraminidase was performed as described by Barton et al. (10).

Nuclear magnetic resonance (NMR) spectroscopy. The 400 MHz spectra of intact gangliosides (4–5 mg) were obtained on a JEOL FX-400 spectrometer in the Fourier transform mode. The operation was performed at 90 C, in dimethyl-d-sulfoxide (Me_2SO-d_6) solution and then in a Me_2SO-d_6 solution containing D_2O at the same temperature. Chemical shifts are given as σ(ppm) from the peak of the internal standard, tetramethylsilane.

Methylation analysis. The linkages between different sugar units were determined by permethylation analysis. The purified gangliosides were permethylated according to the method of Hakomori (11).

The partially methylated alditol acetates were analyzed by gas chromatography-electron impact-mass spectrometry

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Abbreviation: HPTLC, high performance thin layer chromatography.

using a JEOL JMS-HX 100 mass spectrometer. Neutral sugar derivatives of partially methylated alditol acetates were separated on a glass column (2 mm × 200 cm) packed with 3% OV-225 coated on Gaschrom Q at 160–230 C, programmed at 4 C/min, and aminosugar derivatives were separated on a glass column (2 mm × 100 cm) packed with 3% ECNSS-M coated on Chromosorb W (80–100 mesh) at 160–200 C, programmed at 4 C/min.

Analysis of long-chain bases. The long-chain bases were determined by gas liquid chromatography according to the method of Gaver and Sweeley (12) and also by thin layer chromatography. About 100 μg of ganglioside was hydrolyzed in 2.0 N HCl/MeOH at 80 C for three hr. The hydrolysate was mixed with hexane to remove fatty acid methyl esters, and pH was adjusted to 10.0 by the addition of 1.0 N NaOH. The long-chain bases were extracted with hexane. The extract was applied to gas chromatography and also to thin layer chromatography. Dihydrosphingosine, sphingosine and phytosphingosine were used as standards. The detailed analytical conditions of gas chromatography have been described previously (13).

Solvent system for analysis of the long-chain bases by thin layer chromatography was chloroform/methanol/water (60:35:8, v/v/v). The long-chain bases were visualized by spraying the plate (HPTLC plate) with ninhydrine reagent and determined by densitometric analysis.

RESULTS

Isolation of four major gangliosides. Four major resorcinol-positive bands were recognized in HPTLC of the total ganglioside fraction derived from human placenta in solvent system B, as shown in Figure 1. The elution profile of placenta gangliosides from Iatrobeads column chromatography is shown in Figure 2. The four major gangliosides were clearly separated by gradient elution (Fig. 2A). They are designated as G-1 to G-4 from the fastest migrating resorcinol-positive band to the slowest one.

After elution of G-4 ganglioside from the Iatrobeads column, very slowly migrating gangliosides were eluted with the solvent mixture of chloroform/methanol/water (20:80:5, v/v/v). Seven to eight resorcinol-positive spots, including G-4 ganglioside, were separated on HPTLC with solvent system C (Fig. 2B). These gangliosides were rather minor components and were not subjected to structure analysis in the present study.

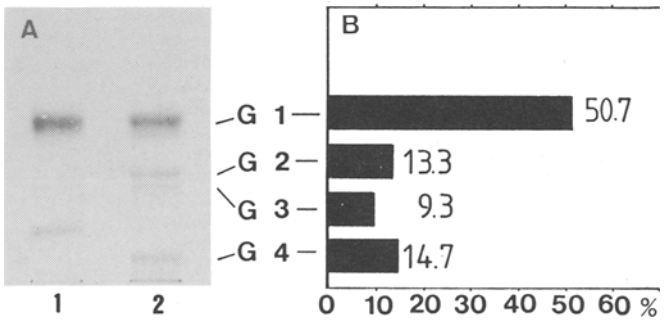

FIG. 1. HPTLC profile (A) and composition (B) of human placenta gangliosides. A, lane 1, GM3; lane 2, gangliosides of human placenta. Solvent B was used for developing. Gangliosides were visualized by spraying with resorcinol reagent.

Each of the four major ganglioside fractions obtained above (Fig. 2A) was compared with standard gangliosides, as shown in Figure 3. G-1 migrated as fast as GM3 obtained from human liver (11) and G-2 showed similar mobility to sialosyllactoneotetraosylceramide obtained from bovine erythrocytes on HPTLC. The G-3 fraction contained small amounts of G-2 and G-4 gangliosides. This component migrated on HPTLC a little more rapidly than i-type ganglioside (IV^{3}NeuGc-lactoneohexaosylceramide) isolated from bovine erythrocytes (14). G-4 ganglioside migrated a little more slowly than I-type ganglioside isolated from bovine erythrocytes (15).

Since fractions G-3 and G-4 were contaminated with other gangliosides, these components were further purified by rechromatography on the Iatrobeads column for structure analysis.

Stepwise degradation of gangliosides by exoglycosidases. Sequential arrangements of sugar in gangliosides were determined by hydrolysis with exoglycosidases followed by identification of the product on HPTLC (Fig. 4, A–D). All gangliosides readily could be hydrolyzed

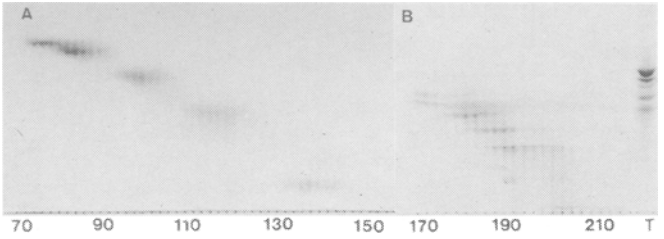

FIG. 2. Elution profile of placenta gangliosides from an Iatrobeads column. A, gangliosides were separated by gradient elution with a solvent mixture of chloroform/methanol/water (83:16:0.5–20:80:5, v/v/v). An aliquot from each fraction was checked by HPTLC with solvent system B. B, higher gangliosides eluted with a mixture of chloroform/methanol/water (20:80:5, v/v/v) after the gradient elution. Gangliosides were developed to the middle of the plate with solvent system C then developed to the top with solvent system A after drying. T, total ganglioside.

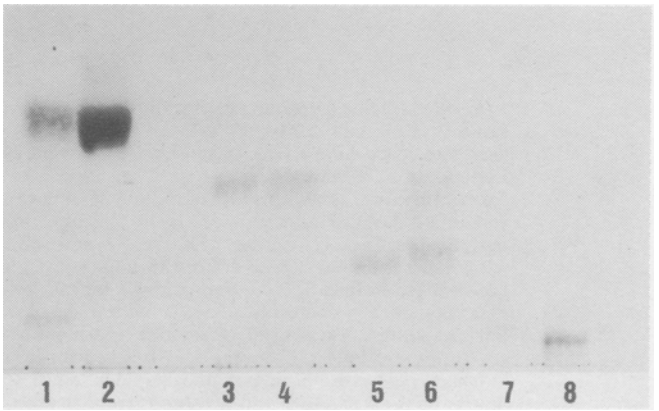

FIG. 3. HPTLC profile of ganglioside fractions separated with Iatrobeads column chromatography. Lanes 1, 3, 5 and 7 are standard gangliosides. Lane 1, GM3; lane 2, G-1; lane 3, sialosylparagloboside (IV^{3}NeuAc-nLc$_4$Cer); lane 4, G-2; lane 5, i-ganglioside (VI^{3}NeuGc-nLc$_6$Cer); lane 6, G-3; lane 7, I-ganglioside (VI^{3}NeuGc-IV^6(II^{3}Gal-nLcNAc)-nLc$_6$Cer); lane 8, G-4. Solvent B was used for developing. Gangliosides were visualized with resorcinol reagent.

to the asialogangliosides by *Cl. perfringens* neuraminidase without addition of detergent. Thus, neuraminic acid seemed to be attached to the terminal sugar component of the gangliosides.

G-1 was converted to a glycolipid with mobility similar to that of lactosylceramide by neuraminidase treatment, and the product was further hydrolyzed to a glycolipid having the same mobility as glucosylceramide by jack bean β-galactosidase treatment. These results indicate that G-1 has the same sugar sequence as GM3 ganglioside (Fig. 4A).

G-2 was hydrolyzed to a glycolipid having the same mobility as lactoneotetraosylceramide on HPTLC by neuraminidase treatment. The product was converted to a glycolipid with the same mobility as lactoneotriaosylceramide by β-galactosidase treatment. Then the product was further converted to lactosylceramide and glucosylceramide by treatment with β-hexosaminidase followed by the β-galactosidase, as shown in Figure 4B. From these observations, G-2 was concluded to have the following anomeric configurations and sugar sequence: sialic acid-βGal-βGlcNAc-βGal-βGlc-ceramide.

G-3 and G-4 were hydrolyzed to lactosylceramide by exoglycosidase treatments in the following order: neuraminidase, β-galactosidase, β-hexosaminidase, β-galactosidase and β-hexosaminidase (Fig. 4C and D). These experimental results indicate that both G-3 and G-4 have a repeating lactosamine unit as their backbone structure. For both G-3 and G-4, the first hydrolysis with β-hexosaminidase after neuramidase and β-galactosidase treatments gave glycolipids having the same mobility as lactoneotetraosyl-ceramide on HPTLC. However, the first neuraminidase treatment and following β-galactosidase treatment of G-3 and G-4 gave glycolipids with different mobilities on HPTLC. Products derived from G-4 migrated much more slowly than those derived from G-3, as shown in Figure 4C and D. These data suggested that G-3 has the following lactosamine repeating unit

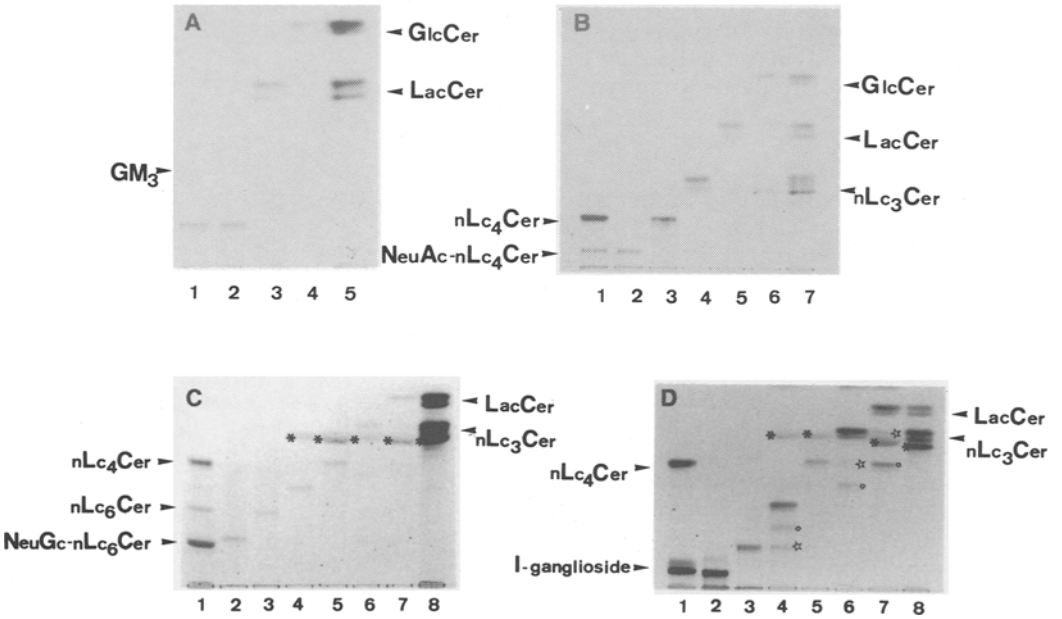

FIG. 4. Stepwise degradation of gangliosides by exoglycosidases. A, enzyme treatment of G-1 ganglioside. Lane 1, GM3; lane 2, G-1; lane 3, G-1 treated with *Cl. perfringens* neuraminidase; lane 4, lipid in lane 3 + β-galactosidase; lane 5, GlcCer and LacCer. Solvent system C was used. B, enzyme treatment of G-2 ganglioside. Lane 1, nLc$_4$Cer and IV^{3}NeuAc-nLc$_4$Cer; lane 2, G-2; lane 3, G-2 + neuraminidase; lane 4, lipid in lane 3 + β-galactosidase; lane 5, lipid in lane 4 + β-hexosaminidase; lane 6, lipid in lane 5 + β- galactosidase; lane 7, GlcCer, LacCer and nLc$_3$Cer from the top. Solvent system C was used. C, enzyme treatment of G-3 ganglioside. Lane 1, nLc$_4$Cer, nLc$_6$Cer, i-ganglioside from bovine erythrocytes; lane 2, G-3; lane 3, G-3 treated with neutraminidase; lane 4, lipid in lane 3 + β-galactosidase; lane 5, lipid in lane 4 + β-hexosaminidase; lane 6, lipid in lane 5 + β-galactosidase; lane 7, lipid in lane 6 + β-hexosaminidase; lane 8, LacCer, nLc$_3$Cer and detergent (sodium taurodeoxycholate). Band of sodium taurodeoxycholate is marked with an asterisk. Solvent system A was used. D, enzyme treatment of G-4 ganglioside. Lane 1, nLc$_4$Cer and I-ganglioside (bovine erythrocytes); lane 2, G-4; lane 3, G-4 + neuraminidase; lane 4, lipid in lane 3 + β-galactosidase; lane 5, lipid in lane 4 + β-hexosaminidase; lane 6, lipid in lane 5 + β-galactosidase; lane 7, lipid in lane 6 + β-hexosaminidase; lane 8, LacCer, nLc$_3$Cer and detergent from the top. Solvent system A was used. Glycolipids were visualized by spraying with orcinol reagent. Maltibands are seen in lane 4, 6 and 7. Bands marked with a circle in lane 4 seemed to be a glycolipid having a following structure: Gal-GlcNAc-Gal-GlcNAc-Gal-Glc-Cer, which was

GlcNAc

a product obtained by the release of one galactose moiety from asialo-G-4 (lipid in lane 3). The lipid marked with a circle in lane 6 should be agalacto-derivative of the lipid marked with a circle in lane 4. A band marked with a circle in lane 7 is the hydrolysis product (nLc$_4$Cer) derived from circle-marked band in lane 6. Bands marked with a star show the rest of the substrate of glycosidase used in each lane.

structure: sialic acid-βGal-βGlcNAc-βGal-βGlcNAc-βGal-βGlc-ceramide. Further, G-4 has the following lactosamine repeating unit structure with branched chain:

sialic acid-βGal-βGlcNAc
\
βGal-βGlcNAc-βGal-βGlc-ceramide.
/
sialic acid-βGal-βGlcNAc

It is quite interesting that no ganglio-series ganglioside could be detected in human placenta.

Proton magnetic resonance (proton NMR) spectra of gangliosides. Proton NMR spectra of the isolated gangliosides taken in Me_2SO-d_6 containing D_2O are shown in Figure 5. Most of the signals were assigned according to the data reported by Koerner et al. (16) and ourselves (17,18). Figure 5A shows that G-1 contained N-acetylneuraminic acid (NeuAc), β-galactose (βGal) and β-glucose (βGlc) in a molar ratio of 1:1:1. G-2 ganglioside was found to contain NeuAc, βGal, βGlcNAc and βGlc in a molar ratio of 1:2:1:1 from the spectrum in Figure 5B.

G-3 ganglioside contained NeuAc, βGal, βGlcNAc and βGlc in a molar ratio of 1:3:2:1 (Fig. 5C).

A characteristic peak at 4.410 ppm appeared in the NMR spectra of G-4 ganglioside (Fig. 5D). It was assigned to the β-anomer proton of N-acetylglucosamine linked to the C-6 position of hexose according to the data reported by Vliegenthart et al. (19) and Dabrowski et al. (20). Other anomeric proton peaks at 4.615, 4.284 ppm and 4.196 ppm were assigned to βGlcNAc, βGal and βGlc, respectively. From the peak areas of the anomeric protons, the molar ratio of sugar components of G-4 was determined to be NeuAc:βGal:βGal(branched position):βGlcNAc:βGlcNAc(1-6R linkage):βGlc=2:3:1:2:1:1.

The peaks appeared between 5.6 and 5.7 in each NMR spectrum were assigned to the protons of double bond of long-chain base. The long-chain bases of these four gangliosides were determined by gas chromatography and also by thin layer chromatography. $C_{18:0}$ Sphingosine base was predominant, and no other sphingosine species was detectable in the four gangliosides.

Methylation analysis. The four separated gangliosides were permethylated and derivatized to partially methylated alditol acetates to analyze the linkage arrangement between sugars. These data are summarized in Table 1. The analytical data indicate that G-1 ganglioside has the same linkage between sugars as GM3 ganglioside. From the methylation analysis together with the enzymatic degradation data and NMR spectra, G-1 ganglioside was determined to be NeuAcα2-3Galβ1-4Glcβ1-1ceramide.

Components of partially methylated alditol acetates derived from G-2 indicated that G-2 had the same sugar linkage as lactoneotetraosylceramide. This result was consistent with the enzymatic degradation data and NMR spectra. Based on these results, G-2 was determined to be NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1ceramide.

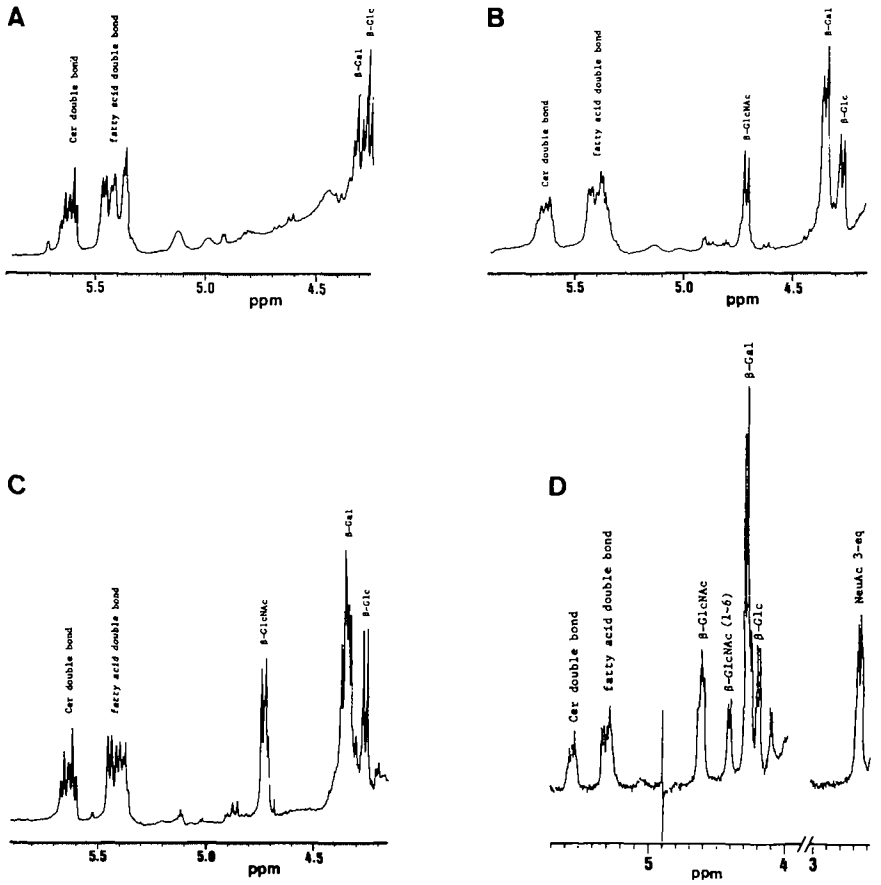

FIG. 5. The 400 MHz proton NMR spectra of placenta gangliosides measured at 90 C in Me_2SO-d_6 containing D_2O solvent. A, G-1 ganglioside; B, G-2 ganglioside; C, G-3 ganglioside; D, G-4 ganglioside.

TABLE 1

Gas Chromatography-Mass Spectrometric Analysis of Partially Methylated Alditol Acetates Derived from the Placenta Gangliosides

Gangliosides	1,4,5-tri-0-Ac-2,3,6-tri-0-Me-glucitol	1,3,5-ti-0-Ac-2,4,6-tri-0-Me-galactitol	1,3,5,6-tetra-0-Ac-2,4-di-0-Me-galactitol	1,4,5-ti-0-Ac-3,6-di-0-Me-glucosaminitol
G-1	+	+	−	−
G-2	+	+	−	+
G-3	+	+	−	+
G-4	+	+	+	+

TABLE 2

Proton Chemical Shifts (ppm) of Placenta Gangliosides Measured at 90 C in D_2O/Me_2SO-d_6

G-1	NeuAcα2-3Galβ1-4Glcβ1-1Cer							
H-1		4.335	4.277					
H-3eq	2.797							
G-2	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer							
H-1		4.363	4.724	4.355	4.278			
H-3eq	2.781							
G-3	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer							
H-1		4.361	4.731	4.336	4.731	4.345	4.258	
H-3eq	2.784							
G-4	NeuAcα2-3Galβ1-4GlcNAcβ1							
	$\backslash$							
	$^{6}_{3}$Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer							
	/							
	NeuAcα2-3Galβ1——— 4GlcNAcβ1							
H-1		4.287	4.410	4.615	4.284	4.615	4.249	4.196
H-3eq	2.740							

In the case of G-3, the partially methylated alditol acetates were the same as those of G-2. As mentioned in connection with the enzymatic degradation study, the presence of a lactosamine repeating unit structure was suggested. From these results and based on the NMR spectra, G-3 was determined to be NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1ceramide.

As compared with the other three gangliosides, G-4 gave a unique derivative of methylated alditol acetate, 2,4-di-0-methyl-1,3,5,6-tetra-0-acetylgalactitol, indicating that this ganglioside has a branching structure at a galactose moiety. This result was consistent with the NMR data. Based on the methylation analysis together with NMR spectra and enzymatic degradation studies, the structure of G-4 was determined to be

NeuAcα2-3Galβ1-4GlcNAcβ1
$\backslash$
$^{6}_{3}$Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1ceramide.
/
NeuAcα2-3Galβ1-4GlcNAcβ1

The NMR peak assignments, listed in Table 2, were consistent with the above structures.

DISCUSSION

Major gangliosides of human placenta were separated and purified, and their structures were determined to be GM3, IV^{3}NeuAc-nLc$_4$Cer, VI^{3}NeuAc-nLc$_6$Cer and VI^{3}NeuAc,IV^6-[II^{3}NeuAc-LcNAc]-nLc$_6$Cer. These gangliosides were characterized as glycolipids containing a lactoneotetraosyl core as the backbone structure, but not a gangliotetraosyl core. Sialosyllactoneohexaosylceramide (i-type ganglioside) and I-type disialoganglioside (VI^{3}NeuAc,IV^6[II^3-NeuAc-LcNAc]-nLc$_6$Cer) were isolated from human erythrocytes (21), and sialosyllactoneohexaosylceramide was separated from chronic myelogenous leukemia cells (22). However, the contents of these components in human erythrocytes are quite low (I-type ganglioside was reported to be 1.8% of total ganglioside of human erythrocytes), and it was very difficult to isolate the pure forms (21). Placenta tissue can be recommended as an excellent source of these lactoneo-series gangliosides.

"Ii" antigens have been characterized as human alloantigens (23–25). I-type ganglioside was detected in adult-type erythrocytes and i-type alloantigen was detected in embryonic erythrocytes (26). I-type ganglioside has been identified as differentiation antigen of human erythrocytes (15,27). In erythrocytes from an embryo and a

newborn baby, i-type erythrocytes are predominant but within a year-and-a-half after birth the erythrocytes have been reported to change from i-type to I-type (26). Further, in an experimental system of human leukemia cell line K-562 (28), expression of i-type changed to I-type accompanying the differentiation of the leukemia cells into erythrocyte-type cells. Similar changes of expression of i and I antigens during differentiation have been demonstrated in mouse myeloid leukemia cells (M1) by Kannagi et al. (29). In the present study, we detected both i- and I-type gangliosides in human placenta, and they are major components in the tissue. "Ii" antigens have been proposed to be precursor substances for ABO-type alloantigens in erythrocytes (15). In the case of erythrocytes, before the transfer of N-acetylneuraminic acid to the terminal galactose of i or I antigen, fucose can be attached to the galactose, and then further addition of α-galactose or α-N-acetylgalactosamine can occur during the development of erythrocytes. However, in the case of placenta before the transfer of fucose to the terminal galactose of i or I antigen, N-acetylneuraminic acid would be transferred to the galactose. This sialylation seems to represent a kind of termination of further glycosylation. This termination signal by transfer of N-acetylneuraminic acid might indicate the termination of gestation (Fig. 6).

Quite recently, we have examined the effect of this I-type ganglioside on the cell growth in a chemically defined medium. Upon addition of the ganglioside, morphological differentiation as well as inhibition of cell growth was observed.

Besides these four major gangliosides, we detected at least five higher gangliosides containing more than 10 sugar residues. Recently, we have separated some of these higher gangliosides by high performance liquid chromatography. This work and the results of a structure study will be reported elsewhere.

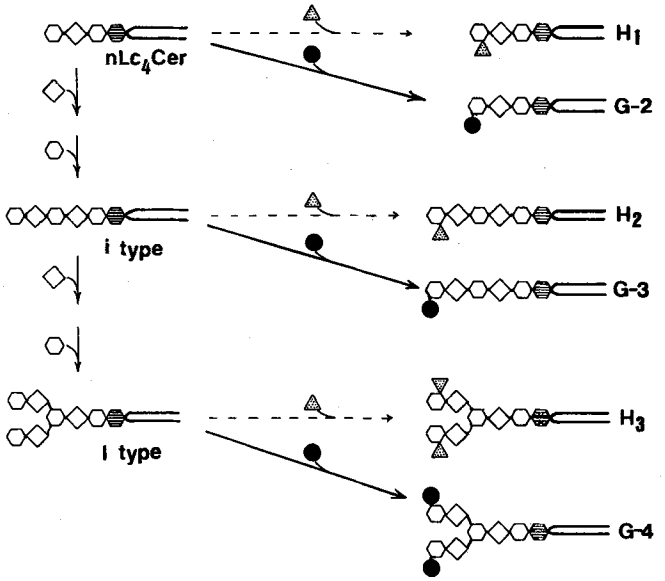

FIG. 6. Proposed metabolic relationship among blood group "Ii," ABO(H)-type and gangliosides in human placenta. In human placenta, G-2, G-3, and G-4 gangliosides were found as characteristic components. Lactoneotetraosylceramide seems to be a precursor ganglioside of these gangliosides. Lactoneotetraosylceramide is converted to i-type glycolipid by the transfer of N-acetylglucosamine (diamond) followed by the transfer of galactose (open hexagon). The i-type glycolipid is converted to I-type lipid by the transfer of N-acetylglucosamine at the C-6 position of the second galactose of i-type lipid, followed by the transfer of galactose. Watanabe et al. (15) proposed that these three glycolipids were converted to blood group AB type lipids through H_1, H_2 and H_3 type lipids by the transfer of fucose to each glycolipid in erythrocytes (indicated by dotted lines). However, in human placenta most of these asialo-type precursor lipids seem to be converted to the respective gangliosides by the transfer of N-acetylneuraminic acid to the terminal galactose. Because fucosylated i- or I-type glycolipids were not detectable in the placenta (data not shown). Since I-type glycolipid has been reported to increase in association with differentiation and development, the appearance of I-type ganglioside in the placenta may correspond to the termination of gestation period. Horizontal fork, ceramide; shaded hexagon, glucose; open hexagon, galactose; diamond, N-acetylglucosamine; shaded circle, n-acetylneuraminic acid; dotted triangle, fucose.

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REFERENCES

1. Hakomori, S. (1981) *Ann. Rev. Biochem. 50,* 733-764.
2. Ando, S. (1983) *Neurochem. Int. 5,* 507-537.
3. Wiegandt, H. (1985) in *New Comprehensive Biochemistry* (Wiegandt, H., ed.) Vol. 10 Glycolipids, pp. 199-260, Elsevier, Amsterdam, New York, Oxford.
4. Hakomori, S. (1984) *Ann. Rev. Immunol. 2,* 103-126.
5. Yogeeswaran, G. (1983) *Adv. Cancer Res. 38,* 289-350.
6. Hakomori, S., and Kannagi, R. (1983) *J. Natl. Cancer Inst. 71,* 231-251.
7. Ledeen, R.W., Yu, R.K., and Eng, L.F. (1973) *J. Neurochem. 21,* 829-839.
8. Chien, J.-L., Li, S.-C., Lain, R., and Li, Y.-T. (1978) *J. Biol. Chem. 253,* 4031-4035.
9. Li, Y.-T., King, M.J., and Li, S.-C. (1980) *Adv. Exp. Med. Biol. 253,* 931-934.
10. Barton, N.W., Lipovac, V., and Rosenberg, A. (1975) *J. Biol. Chem. 250,* 8462-8466.
11. Hakomori, S. (1964) *J. Biochem. Tokyo 55,* 205-208.
12. Gaver, R.C., and Sweeley, C.C. (1966) *J. Am. Chem. Soc. 88,* 3643-3647.
13. Matsumoto, M., Taki, T., Samuelsson, B., Pascher, I., Hirabayashi, Y., Li, S.-C. and Li, Y.-T. (1981) *J. Biol. Chem. 256,* 9737-9741.
14. Nieman, H., Watanabe, K., Hakomori, S., Childs, R.A., and Feizi, T. (1978) *Biochem. Biophys. Res. Commun. 81,* 1286-1293.
15. Watanabe, K., Hakomori, S., Childs, R.A., and Feizi, T. (1979) *J. Biol. Chem. 254,* 3221-3228.
16. Koerner, T.A.W., Prestegard, J.H. Jr., Demou, P.C., and Yu, R.K. (1983) *Biochemistry 22,* 2676-2687.
17. Taki, T., Kimura, H., Gasa, S., Nakamura, M., and Matsumoto, M. (1985) *J. Biol. Chem. 260,* 6219-6225.
18. Taki, T., Hirabayashi, Y., Ishikawa, H., Ando, S., Kon, K., Tanaka, Y., and Matsumoto, M. (1986) *J. Biol. Chem. 261,* 3075-3078.
19. Vliegenthart, J.F.G., Dorland, L., and van Habeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 42,* 209-374.
20. Dabrowski, U., Hanfland, P., Egge, H., Kuhn, S., and Dabrowski, J. (1984) *J. Biol. Chem. 259,* 7648-7651.
21. Kundu, S.K., Samuelsson, B.E., Pascher, I., and Marcus, D. (1983) *J. Biol. Chem. 253,* 13875-13866.
22. Westrich, M.A., Lee, W.M.F., and Macher, B.A. (1983) *Cancer Res. 43,* 5890-5894.
23. Watanabe, K., Powell, M.E., and Hakomori, S. (1978) *J. Biol. Chem. 253,* 8962-8967.
24. Watanabe, K., Powell, M.E., and Hakomori, S. (1979) *J. Biol. Chem. 254,* 8223-8229.

25. Watanabe, K., and Hakomori, S. (1979) *Biochemistry 18,* 5502-5504.
26. Marsh, W.L. (1961) *Br. J. Haematol. 7,* 200-209.
27. Watanabe, K., and Hakomori, S. (1976) *J. Exp. Med. 144,* 644-653.
28. Testa, V., Henri, A., Bettaieb, A., Titeux, M., Vainchecker, W., Tonthat, H., Docklear, M.C., and Rochant, H. (1982) *Cancer Res. 42,* 4624-4630.
29. Kannagi, R., Levery, S.B., and Hakomori, S. (1983) *Proc. Natl. Acad. Sci. USA 80,* 2844-2848.

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Testing a Short-term Feeding Trial to Assess Compositional and Histopathological Changes in Hearts of Rats Fed Vegetable Oils

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Male, female and castrated rats, three wk of age, were fed a low-fat diet for 14 wk followed by high-fat diets (20% by weight) for one wk containing graded levels of erucic acid from 1 to 50%, to evaluate the effect of short-term feeding and interaction of male sex hormones on formation of heart lesions. Some rats within each group were returned to the low-fat diet for one wk after the test period. For comparison, one group of three-wk-old male rats was fed the high fat 50% erucic acid diet for 15 wk. Erucic acid depressed growth rate and food consumption and increased cardiac lipidosis and triglycerides proportional to the erucic acid content of the diet. There were no sex differences, and the effects disappeared once rats were returned to the low-fat diet for one week. There was a significance ($P < 0.05$) in the incidence of myocardial necrosis among male rats fed increased levels of erucic acid for one week, but the response was not linear to the increase in dietary erucic acid. Furthermore, the response was much less than in males fed the 50% erucic acid diet continually for 15 weeks. These results suggest that the short-term model is not a suitable substitute for the long-term feeding trial to test the cardiopathogenicity of a vegetable oil. The significantly lower incidence in myocardial lesions in female and castrated male rats compared with male rats suggests involvement of sex hormones. However, the process appears to be long term, since changes in cardiac lipids and their fatty acid pattern between sexes became evident after one wk on diet but was significant only after long-term feeding.
Lipids 23, 199–206 (1988).

Male Sprague-Dawley rats were shown to be very sensitive to the development of myocardial necrosis; the incidence increased significantly with the amount of fat and erucic acid (22:1n-9) in the diet (1). Previous studies showed that it was necessary to feed weanling rats for at least 16 wk to produce these heart lesions (1–3). Svaar (4) claimed recently that it was not necessary to feed rats for that period of time since the irreversible heart lesions could be produced by feeding adult male rats test diets for only a few days (short-term model). Svaar suggested that myocardial necrosis was due to an interaction of specific fatty acids such as 22:1n-9 with male sex hormones, and he cited the reduction in lesion incidence in castrated male rats but not female rats as evidence in support of his argument.

To test this short-term model, male, female and castrated male rats were fed high-fat test diets for one wk, after 14 wk on a low-fat control diet. The test fat contained graded levels of 22:1n-9 from 1–50%. Some rats were killed after the one-wk feeding period, and the remainder were killed a week later after being placed back on the low-fat control diet. This was done to reduce the myocardial lipidosis known to be associated with the consumption of docosenoic acids (2).

MATERIALS AND METHODS

Diets. The diets were formulated as described (5). The high-fat diet contained 20% by weight rapeseed/canola oil mixtures and a casein/DL-methionine mixture (97/1). The low fat diet contained 5% by weight corn oil and 45% instead of 30% starch. Six dietary oils were used: canola oil (0.7% 22:1n-9), high erucic acid rapeseed (HEAR) oil (49.0% 22:1n-9), and four mixtures of the two rapeseed oils to give a concentration of about 10, 20, 30 and 40% 22:1n-9. Both canola and HEAR oil were obtained from J. D. Jones, Food Research Centre, Agriculture Canada, Ottawa, Ontario, and were of the variety Tower and R-500, respectively. The fatty acid composition of the oils and oil mixtures are shown in Table 1. Two rapeseed oils were mixed to assure that the remaining fatty acids in the oil mixture were similar in composition, i.e. low in saturates, high in 18:3, and intermediate in 18:2.

Animals. A total of 305 male and 40 female weanling Sprague-Dawley rats weighing between 50 and 60 g were purchased from Charles River Canada Inc. (St. Constant,

TABLE 1

Fatty Acid Composition of the Dietary Oils

Fatty acids[a]	Corn oil	Canola oil-HEAR oil mixtures (% 22:1)					
		1%	10%	20%	30%	40%	50%
	(weight, percentage)						
14:0	0.1	0.1	0.1	0.1	0.1	0.1	0.1
16:0	12.3	5.6	4.7	3.9	3.9	3.5	3.2
16:1	0.2	0.3	0.2	0.2	0.2	0.3	0.2
18:0	2.1	2.1	2.1	1.7	1.6	1.3	1.0
18:1n-9	26.1	59.9	53.4	41.5	36.1	25.9	15.5
18:1n-7	0.6	3.0	2.7	2.0	1.7	1.0	0.6
18:2n-6	56.7	19.3	16.9	14.2	12.7	10.9	9.0
18:3n-3	0.6	6.4	6.1	6.4	7.0	8.7	10.6
20:0	0.5	0.5	0.8	0.8	0.8	0.6	0.7
20:1n-9	0.3	1.3	2.2	2.8	3.6	4.2	4.8
20:1n-7		0.1	0.2	0.3	0.5	0.6	0.8
22:0	0.1	0.1	0.4	0.6	0.6	0.6	0.7
22:1n-9		0.7	8.8	22.9	29.0	39.0	49.0
22:1n-7		tr	0.1	0.3	0.4	0.5	0.6
24:0	0.1	0.1	0.2	0.3	0.2	0.2	0.2
24:1n-9		0.1	0.6	1.3	1.1	1.7	2.1

[a]Minor fatty acids such as 15:0, 17:0, 20:2 and 22:2 are not included.

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[1]Deceased.

Abbreviations: C, cholesterol; CE, cholesterol esters; DPG, diphosphatidylglycerol; HEAR, high erucic acid rapeseed; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SP, sphingomyelin; TG, triglycerides; TLC, thin layer chromatography.

Quebec). The animals were weighed, ear-notched for identification, and the males allocated randomly to seven groups of 40 and one group of 25; females were in a separate group. All animals were housed individually. The housing and feeding conditions were described (5). One group of 40 male rats was castrated surgically after four wk on the diet (male c group). Individual body weights were measured every two wk, and every week during diet changes. Data on feed consumption was obtained from groups of five rats per diet by a feed weighback at the end of the 14th, 15th and 16th wk.

Procedures. At the end of the 15th week, 25 rats from each diet were killed by exsanguination while under CO_2 anesthesia. The hearts were removed immediately. Twenty hearts from each diet were fixed in 10% neutral buffered formalin, and the remaining five hearts were extracted immediately for total lipids as described (6). At the end of the 16th week, the remaining 15 rats per diet (except male rats fed HEAR oil continuously) were killed; 10 hearts were used for pathological evaluation, and five were used for lipid analyses.

Three sections from each heart were stained with hematoxylin and eosin, and one section was stained with oil red 0. For details regarding histological procedures, see Charlton et al. (7).

The cardiac lipids were quantitated using the Iatroscan method as described (8). The cardiac lipid classes were separated by three directional TLC (9), removed from the TLC plates, and the lipids were transesterified with anhydrous HCl/CH_3OH (5% w/w). The fatty acid methyl esters were purified by TLC using the developing solvent hexane/diethyl ether (90:10, v/v) before GLC analysis on fused silica capillary columns (10).

Analyses of variance were applied to all measurements, except myocardial necrosis data, for which the log-linear model approach, described by Fienberg (11), was used. Significance of heart lesion data and heart lipid classes was determined at $P < 0.05$ and $P < 0.01$, while the remaining data were assessed at $P < 0.01$ and $P < 0.001$.

RESULTS AND DISCUSSION

Growth. The results in Table 2 confirm the growth depression and reduced feed intake in rats fed diets rich in 22:1n-9 (1,2,12). However, the results were confounded by an increase in body weight as a result of switching from a diet containing 5% fat to one containing 20% fat. As a result, dietary groups with low levels of 22:1n-9 experienced a greater gain in body weight per wk for wk 14 to 15 than for wk 12 to 14, while dietary groups with high levels of 22:1n-9 had similar or decreased body weight gains per week. The growth depressing effect of 22:1n-9 was removed on return to the non-22:1n-9 diet. The weight gain per week of the remaining rats in each

TABLE 2

Body Weight Gain and Feed Intake

Diet group	From: / To:	Body weight gain (g)[a,b] 0 wks / 12 wks	12 wks / 14 wks	14 wks / 15 wks	15 wks / 16 wks	Feed intake (kg/5 rats)[b] 0 wks / 14 wks	14 wks / 15 wks	15 wks / 16 wks
Group size		40	40	40	15	40	40	15
Male—1 wk, 1%		37.9	16.3	26.2	16.2	10.21	0.79	0.77
Male—1 wk, 10%		36.4	17.2	21.2	11.7	10.18	0.74	0.71
Male—1 wk, 20%		37.5	18.7	18.2	15.8	10.14	0.73	0.72
Male—1 wk, 30%		37.1	13.7	17.2	12.9	10.26	0.71	0.72
Male—1 wk, 40%		38.3	17.4	11.1	13.6	10.40	0.70	0.77
Male—1 wk, 50%		37.3	12.7	11.8	14.9	10.20	0.69	0.75
Male c.—1 wk, 50%		31.7	8.4	9.2	8.7	9.63	0.57	0.63
Female—1 wk, 50%		18.5	6.1	4.9	3.8	7.52	0.47	0.48
Male—15 wk, 50%		28.6	22.3	18.0	—	7.87	0.71	—
LSD[c]		2.2	4.4	7.5	7.9	0.16	0.02	0.03
Analyses of variance[d] Source of variation (d.f.)		Mean squares						
Diet[e] (5)		3.4	43.1	228.4**	9.4	0.068	0.0095**	0.0021
Error		2.3	9.7	27.9	54.0	0.136	0.0018	0.0033
Error d.f.		(42)	(39)	(39)	(12)	(42)	(42)	(12)
Sex[f] (2)		752.8**	89.2**	97.9	90.0*	14.6**	0.095**	0.0555**
Error		2.1	11.4	34.9	7.0	0.3	0.001	0.0006
Error d.f.		(21)	(21)	(21)	(6)	(20)	(20)	(6)

[a]Initial body weights of three-wk-old male rats was 47.5 ± 11.2 (n = 305) and female rats was 47.2 ± 4.0 (n = 39).

[b]Body weights and feed intake based on pooled values for groups of five rats.

[c]LSD (least significant difference at $P < 0.01$) based on analysis of variance for six groups of five male rats up to 15th wk, and three groups of five rats from 15th to 16th wk.

[d]Significance levels for F-tests at $P < 0.01$ (*) and $P < 0.001$ (**) level.

[e]Comparison of diets involving male rats fed 1% to 50% 22:1n-9 for one wk.

[f]Comparison of male, castrated male and female rats fed 50% 22:1n-9 for one wk.

group was similar to that prior to the one-wk test period. Female and castrated male rats grew and ate significantly less than entire males irrespective of the diet, which is consistent with previous observations (13).

Myocardial lipidosis. Within one wk of feeding HEAR oil diets, significant myocardial lipidosis was induced in adult rats (Fig. 1). The response was directly related to the dietary level of 22:1n-9. Of the diet sums of squares (162.3), almost all (156.4) were accounted for by the linear trend in 22:1n-9. These results are in agreement with those previously reported (2,4). There was no significant difference ($P > 0.05$) in severity between adult male, female and castrated male rats fed the same diet similar to that observed in young rats (14–16). Continuous feeding of the same HEAR oil diet reduced the severity of myocardial lipidosis significantly ($P < 0.01$) compared with rats fed the diet for only one wk (Fig. 1), which is consistent with the finding that maximum myocardial lipidosis occurs after one wk (2). The relatively high residual lipidosis in males fed HEAR oil for 15 wk may be due to the high dietary concentration of 22:1n-9.

Substitution of the diets containing 22:1n-9 with corn oil for one wk reduced myocardial lipidosis dramatically, as shown (2). No significant differences in lipidosis were observed between adult male, female and castrated male rats, although females had a slightly lower incidence, similar to that observed in young rats (16). The switch also involved a reduction in the level of fat in the diet from 20% to 5% by weight, which might have expedited the regression of myodardial fat infiltration.

Myocardial necrosis. The incidence and severity of myocardial necrosis was not significantly different in rats examined after the 15th and 16th wk, and for this reason the results were combined in Table 3.

Among males fed increasing levels of 22:1n-9 for one wk, there was a significant difference in incidence ($P < 0.05$) but not severity of myocardial necrosis. The response to dietary 22:1n-9, however, was not linear. These results are not consistent with those reported by Svaar (4), who observed a good correlation between the dietary concentration of 22:1n-9 and the severity of heart lesions in male rats after one wk. In fact, it was for this reason that Svaar (4) suggested that a one-wk feeding to adult male rats (short-term model) could be used to test the cardiopathogenicity of a dietary oil, instead of the usual 16-wk feeding period. It appears highly unlikely that the difference in strains of rats used (Wistar [4] vs Sprague-Dawley) would account for the discrepancy, since the Sprague-Dawley male rat used in this study is known to be more sensitive to the development of myocardial necrosis than the Wistar male rat (1,17). Our results therefore would suggest that a one-wk feeding period to adult male rats would not lead to a conclusive evaluation of the cardiopathogenicity of a vegetable oil.

Long-term feeding of the same diet (50% 22:1n-9) resulted in a much higher incidence and severity of

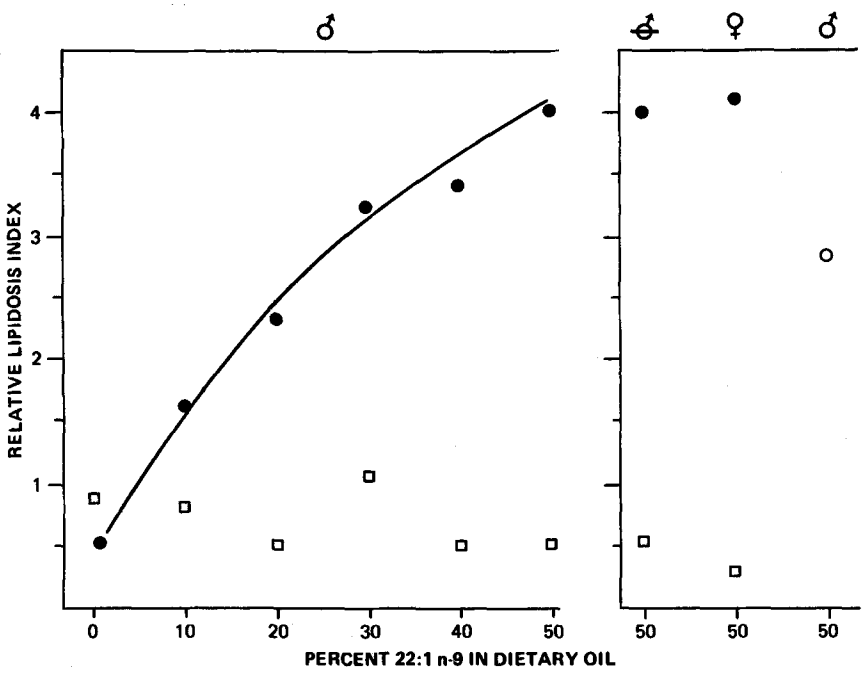

FIG. 1. Mean myocardial lipidosis scores of male rats (n = 20) fed the experimental diets containing graded levels of erucic acid (22:1n-9) from 1 to 50% for one wk (●). An additional 10 rats in each group were examined a week later after having been returned to the 5% corn oil diet (□). Castrated male (♂-) and female rats (n = 20) also were fed an experimental diet containing 50% 22:1n-9 for one wk (●), and an additional 10 rats in each group were fed a 5% corn oil diet for another week (□). One group of male rats (n = 20) were fed a rapeseed oil containing 50% 22:1n-9 for 15 wk (○). Lipidosis scores as determined by oil red 0: 0, no fat stain; 1, very slight; 2, slight; 3, moderate; 4, marked; 5, severe.

TABLE 3

Myocardial Necrosis in Sprague-Dawley Rats Fed the Experimental Diets for 15 and 16 Weeks[a]

Diet group	Incidence	Severity index[b]			
	Affected/examined	1	2	3-6	>6
Male—1 wk, 1%	7/30	5	2	0	0
Male—1 wk, 10%	9/30	6	2	1	0
Male—1 wk, 20%	17/30	10	2	4	1
Male—1 wk, 30%	15/30	9	2	4	0
Male—1 wk, 40%	9/30	7	1	1	0
Male—1 wk, 50%	16/30	8	4	4	0
Male c.—1 wk, 50%	7/30	5	1	1	0
Female—1 wk, 50%	5/30	3	1	1	0
Male—15 wk, 50%	20/20	0	0	2	18
χ^2 Analysis[c]					
Comparisons (d.f.)	Incidence	Severity[d]			
All 1-wk diets (7)	22.37**	2.71			
Diet[e] (5)	13.07*	2.43			
Sex[f] (2)	10.51**	0.96			

[a]Twenty rats of each group were examined histologically for H&E staining after 15 wk; an additional 10 rats were examined from all diets at 16 wk. The results of both time periods were combined because these were not significantly different.

[b]Severity of myocardial necrosis represents the number of rats with one, two, three to six, and > six lesions per heart (3 sections/heart were examined).

[c]The χ^2 analysis was according to a method described by Fienberg (11); d.f., degrees of freedom; and significance levels at $P < 0.05$ (*) and $P < 0.01$ (**).

[d]For statistical analysis of severity two groups were compared, rats with one lesion vs rats with more than one lesion.

[e]Comparison of male rats fed for one wk diets containing 1 to 50% 22:1n-9.

[f]Comparison of male, castrated male and female rats fed for one wk the diet containing 50% 22:1n-9.

myocardial necrosis than the one-wk trial (Table 3). These results suggest that long-term feeding will be necessary in any case to differentiate fats and oils of similar cardiopathogenic response.

The suggestion has been made that the cardiac injury might be due to an interaction of male sex hormones with specific dietary fat components, such as 22:1n-9 (4,13). The lower incidence of myocardial necrosis in female (15,18,19) and castrated male rats (13), compared with males (Table 3), would support this view. However, even though all three groups ate the same diet, their growth rate and feed consumption were markedly different (males > castrated males > females), see Table 2. The effect of differences in growth and feed consumption cannot be ignored in view of the following evidence. First, for a particular diet light male rats showed significantly ($P < 0.01$) less myocardial necrosis than heavier rats (20). Second, male rats that ate similar diets but weighed less after the same feeding period, had a lower incidence of heart lesions (21). Third, in one study castrating Sprague-Dawley male rats had no effect on growth, and the incidence of myocardial necrosis was similar (22). It is this evidence that suggests that both sex hormones and growth rate are involved in the etiology of myocardial necrosis in rats. The relative contribution of these two factors, however, is impossible to separate at this time.

Cardiac lipid classes. Only the data from selected diets were analyzed to determine if diet or sex resulted in cardiac lipid changes that might reflect the observed cardiopathological responses.

There were only a few sex differences in cardiac lipids after feeding the HEAR oil diet for one week (Table 4). However, a common trend was observed. Male rats had a higher concentration of cardiac phospholipids (DPG, PE, PC, and SP) than either females or castrated males; the latter two groups were similar. Prolonged feeding (15 wk) of the same HEAR oil diet resulted in a further increase of these cardiac phospholipids. On the other hand, male rats fed the diet low in 22:1n-9 had a phospholipid composition more like that of female and castrated male rats. These results suggest that an increase in all cardiac phospholipids could be related to an increase in the incidence and severity of myocardial necrosis. The cardiac lipid changes already were evident after one wk but were more pronounced on long-term feeding. Some of the published reports showed an increase in either PC (23), DPG (24) or SP (25) when male rats were fed diets rich in 22:1n-9, but the results had not been consistent nor shown for all cardiac phospholipids (26).

There was no difference between sexes in the amount of cardiac TG accumulated within one wk in adult Sprague-Dawley rats (Table 4). Female hearts contained slightly lower levels of cardiac TG than males or castrated males, but this was not statistically significant. These results are in marked contrast with the only other study in which sex differences were investigated and young Wistar males were shown to have a 3.5 times higher cardiac TG level than females fed for three days a partially hydrogenated fish oil containing 14.5% 22:1 isomers (27). It does not appear that differences in age and strain of rat, diet (type of 22:1 isomer), or length of feeding could explain these large differences. Furthermore, the unusually low values of cardiac TG in their results (8.3 to 47.5 nmol/g of heart) (27) are about 1/100th of those reported by others (6,28).

Triglycerides. The fatty acid composition of cardiac TG (Table 5) was not significantly different among the three sex groups (males, castrated males and females) fed the same HEAR oil (50% 22:1 n-9) diet for one wk. Thomassen et al. (27) also reported no sex difference in the fatty acid composition of the accumulated TG, despite a 3.5-fold higher level of cardiac TG in male compared with female weanling Wistar rats fed fish oil high in 22:1n-11.

Continued feeding of a diet rich in 22:1n-9 may have increased the rat's capacity to metabolize 22:1n-9 as evidenced by a lower level of 22:1n-9 and higher levels of 20:1n-9 and 18:1n-9 compared with male rats fed the same diet for only one wk (Table 5). These products were shown to be formed by peroxisomal β-oxidation of 22:1n-9 in rat heart (29,30) and liver (31). The results from this study suggest that the metabolism of the long chain monounsaturated fatty acids, even in adult rats, is slow and very similar between sexes.

Phospholipids. It is not the intent in this study to discuss the characteristic differences between cardiac phospholipids, but simply to identify differences that could be related to differences in cardiopathogenic response. Many of the differences between the Canola and

HEAR oil group were related to dietary monounsaturated fatty acids (18:1, 20:1 and 22:1), and thus provide no critical evaluation (Table 6). On the other hand, male, female and castrated male rats, fed the same diet for one wk, might yield compositional differences that could be related to known sex differences in cardiopathogenic response.

In general, there were few sex differences, and these were found mainly in the composition of PI and DPG (Table 6). Castrated males differed in their cardiac fatty

TABLE 4

Heart Weight and Cardiac Lipid Composition

Diet group	Heart weight	Lipid classes[a]							
		CE	TG	C	DPG	PE	PS&PI	PC	SP
	(g)	(mg/g wet heart weight)							
Male—1 wk, 1%	1.40	0.5	3.6	0.5	2.0	4.5	0.4	8.2	0.4
Male—1 wk, 50%	1.31	0.6	11.1	0.6	2.4	4.5	0.4	9.5	0.6
Male c.—1 wk, 50%	1.21	0.5	11.5	0.5	2.0	3.5	0.3	8.0	0.3
Female—1 wk, 50%	0.94	0.5	10.0	0.5	1.9	3.7	0.4	7.6	0.3
Males—15 wk, 50%	1.47	0.6	6.4	0.6	2.7	4.9	0.5	10.9	0.6
LSD[b]	0.2	0.2	7.9	0.2	0.5	0.8	0.4	2.3	0.3
Analyses of variance Sources of variation (d.f.)[c]		Mean squares							
All groups (4)	0.35**	0.04	117*	0.04	0.93**	3.4**	0.04	15.2**	0.13
Sex[d] (2)	0.29**	0.03	5	0.03	0.58*	2.8*	0.02	6.7	0.15
Diet[e] (1)	0.04	0.04	287*	0.02	0.60*	0.01	0.01	8.0	0.21
Error (19)	0.03	0.03	36	0.02	0.13	0.4	0.09	3.0	0.05

[a]CE, cholesterol ester; TG, triglyceride; C, cholesterol; DPG diphosphatidylglycerol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI , phosphatidylinositol; PC, phosphatidylcholine; SP, sphingomyelin. All values are means of five rats/diet and two analyses/rat.

[b]LSD, least significant differences at $P < 0.05$.

[c]d.f., degrees of freedom; significance at $P < 0.05$ (*) and $P < 0.01$ (**).

[d]Comparison of male, castrated male and female rats fed 50% 22:1n-9 for one wk.

[e]Comparison of entire males fed diets containing either 1 or 50% 22:1n-9.

TABLE 5

Fatty Acid Composition of Cardiac Triglycerides

Fatty acids	Male 1 wk 1%	Male 1 wk 50%	Male c. 1 wk 50%	Female 1 wk 50%	Male 15 wk 50%	LSD	Effect of[a]		
							Sex	Canola	HEAR
	(weight percentage)								
14:0	0.9	1.0	0.6	0.7	0.5	0.5	NS	NS	NS
16:0	23.3	18.4	13.7	15.1	13.8	8.3	NS	*	NS
16:1n-7	0.6	1.6	0.8	0.9	0.9	0.9	NS	NS	NS
18:0	5.7	4.1	3.5	4.0	3.8	1.3	NS	**	NS
18:1n-9	36.0	24.5	26.8	27.4	34.9	4.8	NS	**	**
18:1n-7	4.6	2.5	2.3	1.8	1.6	0.9	NS	NS	NS
18:2n-6	18.7	10.8	12.4	9.5	9.5	4.0	NS	**	NS
18:3n-3	1.2	1.0	1.5	1.3	4.5	0.7	NS	NS	**
20:0	0.3	0.3	0.4	0.3	0.4	0.1	NS	NS	NS
20:1n-9	1.1	3.7	4.5	4.1	7.4	1.3	NS	**	**
20:2n-6	0.2	0.2	0.3	0.3	0.3	0.1	NS	NS	NS
20:4n-6	2.1	0.7	1.2	0.8	0.7	0.3	*	**	NS
22:0	0.1	0.3	0.5	0.3	0.3	0.2	NS	**	NS
22:1n-9	0.4	20.7	26.4	21.0	17.7	11.6	NS	**	NS
ΣC22 PUFA	1.3	0.5	1.0	0.6	0.9	0.5	NS	*	NS

Abbreviations: C, castrated; LSD, least significant differences ($P < 0.01$); Canola, canola oil; HEAR, high erucic acid rapeseed; PUFA, polyunsaturated fatty acids; NS, not significant at $P > 0.01$; significant at $P < 0.01$ (*) and $P < 0.001$ (**).

[a]Statistics were based on analysis of variance of pooled data. Sex comparison was among the three groups of rats (males, castrated males and females) fed the HEAR oil diet (50% 22:1n-9) for one wk. The Canola oil comparisons were between males fed the canola oil (1% 22:1n-9) for one wk and the HEAR oil (50% 22:1n-9) for 15 wk; the HEAR oil comparison was between the three groups of rats (male, castrated males and females) fed the HEAR oil diet for one wk.

TABLE 6

Fatty Acid Composition of the Major Cardiac Phospholipids[a]

Lipid classes diet	DMA	Saturates 16:0	18:0	Σ Sat.	Monounsaturates 18:1 n-9	18:1 n-7	20:1 n-9	22:1 n-9	Polyunsaturates (PUFA) 18:2 n-6	20:4 n-6	ΣC22 n-3	ΣC22 PUFA
					(weight percentage)							
Phosphatidylcholine (PC)												
Male—1 wk, 1%	0.8	16.3	31.4	48.3	6.8	5.3	0.3	<0.1	7.2	26.1	2.2	3.9
Male—1 wk, 50%	0.8	13.1	31.7	45.3	6.6	3.4	1.3	1.6	7.3	29.3	1.3	2.4
Male c.—1 wk, 50%	0.7	12.7	32.3	45.7	6.3	2.5	1.5	1.9	5.7	30.8	1.6	2.9
Female—1 wk, 50%	0.9	13.8	32.4	46.8	7.7	2.7	1.1	1.5	8.3	26.3	1.2	2.6
Male—15 wk, 50%	0.6	13.1	30.5	44.2	9.2	2.4	2.1	1.9	11.0	21.7	3.8	3.8
LSD	0.2	3.1	6.9	9.0	1.0	0.9	0.4	0.5	1.9	7.4	0.8	1.4
Phosphatidylethanolamine (PE)												
Male—1 wk, 1%	8.7	6.1	24.5	31.5	6.0	3.6	0.2	<0.1	5.0	21.7	10.7	21.9
Male—1 wk, 50%	9.1	5.5	22.0	27.9	8.0	2.9	1.4	1.5	4.9	23.9	9.0	18.6
Male c.—1 wk, 50%	8.8	5.1	21.6	27.8	7.7	2.1	1.5	1.7	3.4	22.9	11.2	22.0
Female—1 wk, 50%	9.9	5.6	26.6	32.7	8.0	2.2	1.3	1.4	4.4	20.6	7.9	17.8
Male—15 wk, 50%	7.6	5.6	23.5	29.8	9.2	1.9	2.2	2.2	4.7	18.3	20.8	21.1
LSD	5.1	1.7	4.0	3.3	1.4	0.7	0.5	0.6	1.0	5.5	2.9	6.1
Phosphatidylinositol (PI)												
Male—1 wk, 1%	—	5.1	44.5	51.3	9.7	1.6	0.3	0.2	4.5	27.2	1.3	2.3
Male—1 wk, 50%	—	3.6	51.4	56.0	10.6	1.5	1.0	1.5	4.8	20.3	1.2	2.6
Male c.—1 wk, 50%	—	4.9	41.6	48.4	12.5	1.3	1.2	1.7	3.7	24.1	2.0	3.4
Female—1 wk, 50%	—	4.7	47.6	53.4	15.4	1.6	1.2	1.4	5.5	17.1	0.9	2.1
Male—15 wk, 50%	—	4.3	42.7	48.8	10.8	1.0	1.4	1.7	4.2	24.6	2.8	2.9
LSD		1.9	3.2	3.8	2.0	0.5	0.5	1.0	1.5	3.5	1.1	1.8
Phosphatidylserine (PS)												
Male—1 wk, 1%	—	2.9	51.7	57.3	4.1	0.7	0.4	0.5	1.4	8.0	9.6	25.0
Male—1 wk, 50%	—	3.8	53.4	58.9	4.9	1.1	1.2	5.1	2.1	8.5	5.7	15.5
Male c.—1 wk, 50%	—	8.1	37.1	50.2	7.4	0.7	1.0	5.8	1.8	6.7	8.1	19.0
Female—1 wk, 50%	—	5.3	54.7	61.9	6.1	0.9	1.0	4.4	2.5	6.8	4.3	13.9
Male—15 wk, 50%	—	4.0	41.8	48.4	6.7	0.7	1.8	8.2	2.6	6.4	20.0	20.6
LSD		4.8	9.7	10.5	2.6	0.4	0.5	1.8	1.1	3.4	5.5	7.9
Diphosphatidylglycerol (DPG)												
Male—1 wk, 1%	—	1.2	1.7	3.3	4.9	3.9	0.1	<0.1	78.1	3.3	0.9	2.8
Male—1 wk, 50%	—	1.0	1.6	2.9	6.1	2.2	0.8	1.6	76.9	2.6	0.5	1.8
Male c.—1 wk, 50%	—	1.3	2.3	4.2	8.1	3.2	1.1	2.8	66.0	5.2	1.2	3.7
Female—1 wk, 50%	—	1.8	1.8	4.3	6.8	2.0	0.8	1.5	75.2	2.2	0.5	2.0
Male—15 wk, 50%	—	1.2	1.6	3.2	7.2	2.2	1.7	5.8	65.4	2.6	2.8	2.8
LSD		0.7	1.2	1.9	1.9	1.4	0.4	1.0	5.9	0.9	0.9	2.5
Statistics												
Effect of sex PC	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS
PE	NS	NS	NS	*	NS	NS	NS	NS	*	NS	NS	NS
PI	—	NS	**	**	**	NS	NS	NS	NS	*	NS	NS
PS	—	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
DPG	—	NS	NS	NS	NS	NS	NS	*	*	**	*	NS
Effect of LEAR PC	NS	*	NS	NS	NS	**	**	**	NS	NS	NS	NS
oil PE	NS	NS	NS	NS	**	**	**	**	NS	NS	NS	NS
PI	—	NS	NS	NS	**	NS	**	**	NS	**	NS	NS
PS	—	*	NS	NS	NS	NS	**	**	NS	NS	NS	**
DPG	—	NS	NS	NS	**	*	**	**	*	NS	NS	NS
Effect of HEAR PC	*	NS	NS	NS	**	NS	**	NS	*	*	**	NS
oil (15 wk) PE	NS	NS	NS	NS	*	NS	**	**	NS	*	**	NS
PI	—	*	*	**	*	*	NS	NS	NS	*	**	NS
PS	—	NS	NS	*	NS	NS	**	**	NS	NS	**	NS
DPG	—	NS	NS	NS	NS	NS	**	**	**	NS	**	NS

[a]See footnote, Table 5.

[b]DMA, dimethylacetals.

acid composition of PI and DPG from both males and females, while the latter two groups were similar in composition. This was unexpected, since the cardiopathogenic response showed a similarity between castrated males and females, and both were significantly different from males (Table 3). Likewise, the incorporation of 22:1n-9 into cardiac phospholipids showed little evidence of being related to the incidence of myocardial necrosis. Although there were no significant sex differences in 22:1n-9 accumulation in cardiac phospholipids (except DPG), the trend was always the same, females < males < castrated males. This suggests that 22:1n-9 was not solely responsible for the development of myocardial necrosis, as concluded (32).

Previous studies showed that the incidence of myocardial necrosis in male rats was inversely related to the concentration of dietary saturates and directly related to the concentration of dietary 18:3n-3 (5,33,34). The heart lipids had increased levels of saturates and a high level of C22n-3 PUFA in all cardiac phospholipids (except DPG), which correlated well with the incidence of heart lesions in male rats (35). In this study, the differences in content of saturates and C22n-3 PUFAs in cardiac lipids between sexes was occasionally significant (Table 6), but did not parallel the observed incidence of myocardial necrosis (Table 3). Castrated rats generally were different from male and female rats. It should be noted, however, that male rats fed the 50% 22:1n-9 diet for 15 weeks had significantly higher levels of C22n-3 PUFA in all cardiac phospholipids and generally lower levels of saturates. These results indicate that the fatty acid composition was slower to change in adult rats (this study) than in weanling rats (35) fed similar diets for one wk. This in turn might explain the relatively low severity of myocardial necrosis observed in adult rats fed the HEAR oil diets for one wk compared with feeding the diet for 15 wk.

Sphingomyelin. Male rats fed the 50% 22:1n-9 diet for one wk had a reduced level of saturated fatty acids in cardiac SP, and this reduction was significant on continuous feeding (15 wk) of the HEAR oil diet (Table 7). Saturated fatty acids containing 20 or more carbon atoms decreased while long-chain monounsaturates (18:1n-9, 22:1n-9 and 24:1n-9) increased in male rats fed HEAR oil for one week, and even more so when the male rats were fed for 15 wk. The results were consistent (females < castrated males < males—1 wk < males—15 wk), and the trend paralleled the observed incidence of myocardial necrosis. Even though SP is not a major component of cardiac phospholipids, its influence might be significant, since it is present in selected membranes such as the outer membrane of mitochondria (36) and it has the highest net incorporation of 22:1n-9 among cardiac phospholipids. The net incorporation includes 22:1n-9 itself (~7%), plus the increase in 24:1n-9 (~12%) above levels that occur in rats fed diets such as corn or soybean oils (37), since 24:1n-9 is the product of chain elongation of 22:1n-9 (38–40).

The results of this study indicate that the well-known, high incidence of myocardial necrosis in male rats fed HEAR oil diets (41) could not be duplicated by a one-wk feeding, even when the animals were fed during the age of sexual maturation (presumed sensitive period). Based on this study, a much larger number of rats and a high level of test oil in the diet will be required to obtain a more conclusive answer regarding the cardiopathogenicity of an oil, if the one-wk feeding period proposed by Svaar (4), was implemented. Furthermore, such a short-term feeding study might not provide information as to the cause and development of myocardial necrosis from non-brassica vegetable oils.

Our results suggest that we might not be dealing with an acute cardiopathogenic agent(s) in vegetable oils, but rather with a nutritional problem in the rat, specifically

TABLE 7

Fatty Acid Composition of Cardiac Sphingomyelin[a]

Fatty acids	Male 1 wk 1%	Male 1 wk 50%	Male c. 1 wk 50%	Female 1 wk 50%	Male 15 wk 50%	LSD	Effect of Sex	Effect of Canola	Effect of HEAR
				(weight percentage)					
14:0	0.4	0.7	0.7	0.8	0.3	0.6	NS	NS	NS
15:0	0.4	0.4	0.6	0.6	0.5	0.7	NS	NS	NS
16:0	12.9	15.6	13.9	13.9	13.0	3.4	NS	NS	NS
16:1n-7	1.0	2.0	2.0	2.0	1.0	2.1	NS	NS	NS
17:0	0.5	0.7	0.6	0.8	0.5	0.4	NS	NS	NS
18:0	19.8	16.1	18.2	16.3	24.2	3.6	NS	*	**
18:1n-9	4.2	5.6	4.9	3.7	6.2	2.3	NS	NS	NS
20:0	19.3	11.1	10.8	11.8	8.2	2.0	NS	**	*
21:0	0.8	0.6	0.6	0.6	0.3	0.2	NS	*	**
22:0	14.9	14.1	16.3	17.4	15.6	3.5	NS	NS	NS
22:1n-9	0.3	5.2	4.3	3.3	6.8	2.0	NS	**	*
23:0	3.0	2.5	2.6	2.8	0.8	0.5	NS	NS	*
24:0	10.7	8.8	9.3	9.9	3.3	2.3	NS	NS	**
24:1n-9	10.6	13.9	14.1	10.8	18.1	3.4	NS	NS	**
ΣSaturates	82.8	70.7	73.6	75.0	66.8	5.7	NS	**	*

[a]See footnotes, Table 5.

in fast-growing albino male rats (20). The development of myocardial necrosis, although not totally understood, appears to be caused by a combination of factors such as an alteration of cardiac phospholipids and/or their fatty acid composition (saturates, C22n-3 PUFAs, and long-chain monoenes of 20:1, 22:1 and 24:1). The compositional changes were evident after one wk on these experimental diets but much more so after continued feeding.

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REFERENCES

1. Kramer, J.K.G., and Sauer, F.D. (1983) in *High and Low Erucic Acid Rapeseed Oils* (Kramer, J.K.G., Sauer, F.D., and Pigden, W.J., eds.) pp. 413-474, Academic Press, New York, NY.
2. Abdellatif, A.M.M., and Vles, R.O. (1970) *Nutr. Metab. 12,* 285-295.
3. Beare-Rogers, J.L., and Nera, E.A. (1972) *Comp. Biochem. Physiol. 41B,* 793-800.
4. Svaar, H. (1982) in *Nutritional Evaluation of Long-Chain Fatty Acids in Fish Oil* (Barlow, S.M., and Stansby, M.E., eds.) pp. 163-184, Academic Press, London.
5. Farnworth, E.R., Kramer, J.K.G., Thompson, B.K., and Corner, A.H. (1982) *J. Nutr. 112,* 231-240.
6. Kramer, J.K.G., and Hulan, H.W. (1978) *J. Lipid Res. 19,* 103-106.
7. Charlton, K.M., Corner, A.H., Davey, K., Kramer, J.K.G., Mahadevan, S. and Sauer, F.D. (1975) *Can. J. Comp. Med. 39,* 261-269.
8. Kramer, J.K.G., Farnworth, E.R., and Thompson, B.K. (1985) *Lipids 20,* 536-541.
9. Kramer, J.K.G., Fouchard, R.C., and Farnworth, E.R. (1983) *Lipids 18,* 896-899.
10. Kramer, J.K.G., Fouchard, R.C., and Jenkins, K.J. (1985) *J. Chromatogr. Sci. 23,* 54-56.
11. Fienberg, S.E. (1970) *Ecology 51,* 419-433.
12. Thomasson, H.J., and Boldingh, J. (1955) *J. Nutr. 56,* 469-475.
13. Hulan, H.W., Kramer, J.K.G., Corner, A.H., and Thompson, B.K. (1977) *Can. J. Physiol. Pharmacol. 55,* 265-271.
14. Beare-Rogers, J.L., Nera, E.A., and Heggtveit, H.A. (1971) *Can. Inst. Food Technol. J. 4,* 120-124.
15. Kramer, J.K.G., Mahadevan, S., Hunt, J.R., Sauer, F.D., Corner, A.H., and Charlton, K.M. (1973) *J. Nutr. 103,* 1696-1708.
16. Engfeldt, B., and Brunius, E. (1975) *Acta Med. Scand. Suppl. 585,* 15-26.
17. Vles, R.O. (1978) *Rev. Fr. Corps Gras 25,* 289-295.
18. Rocquelin, G., and Cluzan, R. (1968) *Ann. Biol. Anim. Biochim. Biophys. 8,* 395-406.
19. Nolen, G.A. (1981) *J. Am. Oil Chem. Soc. 58,* 31-37.
20. Thompson, B.K., Kramer, J.K.G., Farnworth, E.R., Corner, A.H., and Hulan, H.W. (1984) *Nutr. Res. 4,* 681-689.
21. Kramer, J.K.G., Hulan, H.W., Corner, A.H., Thompson, B.K., Holfeld, N., and Mills, J.H.L. (1979) *Lipids 14,* 773-780.
22. Vles, R.O. (1979) *Proc. La Nouvelle Huile de Colza,* Centre National de Coordination des Études et Recherches sur la Nutrition et l'Alimentation, Paris, France, pp. 149-156.
23. Ray, S., Sen Gupta, K.P., and Chatterjee, G.C. (1979) *Indian J. Exp. Biol. 17,* 918-921.
24. Rocquelin, G., Juaneda, P., and Cluzan, R. (1981) *Ann. Nutr. Metab. 25,* 350-361.
25. Dewailly, P., Sezille, G., Nouvelot, A., Fruchart, J.C., and Jaillard, J. (1977) *Lipids 12,* 301-306.
26. Kramer, J.K.G., and Sauer, F.D. (1983) in *High and Low Erucic Acid Rapeseed Oils* (Kramer, J.K.G., Sauer, F.D., and Pigden, W.J., eds.) pp. 475-513, Academic Press, New York, NY.
27. Thomassen, M.S., Norseth, J., and Christiansen, E.N. (1985) *Lipids 20,* 668-674.
28. Stam, H., Geelhoed-Mieras, T., and Hülsmann, W.C. (1980) *Lipids 15,* 242-250.
29. Christiansen, R.Z., Norseth, J., and Christiansen, E.N. (1979) *Lipids 14,* 614-618.
30. Norseth, J., and Thomassen, M.S. (1983) *Biochim. Biophys. Acta 751,* 312-320.
31. Norseth, J., and Christophersen, B.O. (1978) *FEBS Lett. 88,* 353-357.
32. Hulan, H.W., Kramer, J.K.G., and Corner, A.H. (1977) *Lipids 12,* 951-956.
33. Trenholm, H.L., Thompson, B.K., and Kramer, J.K.G. (1979) *Can. Inst. Food Sci. Technol. J. 12,* 189-193.
34. Kramer, J.K.G., Farnworth, E.R., Thompson, B.K., Corner, A.H., and Trenholm, H.L. (1982) *Lipids 17,* 372-382.
35. Kramer, J.K.G., Farnworth, E.R., and Thompson, B.K. (1985) *Lipids 20,* 635-644.
36. Colbeau, A., Nachbaur, J., and Vignais, P.M. (1971) *Biochim. Biophys. Acta 249,* 462-492.
37. Kramer, J.K.G. (1980) *Lipids 15,* 651-660.
38. Kishimoto, Y., and Radin, N.S. (1963) *J. Lipid Res. 4,* 444-447.
39. Bourre, J.-M., Daudu, O., and Baumann, N. (1976) *Biochim. Biophys. Acta 424,* 1-7.
40. Lecerf, J. (1980) *Biochim. Biophys. Acta 617,* 398-409.
41. Sauer, F.D., and Kramer, J.K.G. (1983) in *High and Low Erucic Acid Rapeseed Oils* (Kramer, J.K.G., Sauer, F.D., and Pigden, W.J., eds.) pp. 253-292, Academic Press, New York, NY.

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([3]H)Forskolin- and ([3]H)Dihydroalprenolol-binding Sites and Adenylate Cyclase Activity in Heart of Rats Fed Diets Containing Different Oils

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The characteristics of the cardiac adenylate cyclase system were studied in rats fed diets containing fish oil (menhaden oil) and other oils. Adenylate cyclase activity generally was higher in cardiac homogenates and membranes of rats fed diet containing 10% menhaden oil than in the other oils. The increase in enzyme activity, especially in forskolin-stimulated activity, was associated with an increase in the concentration of the [3H]forskolin-binding sites in cardiac membranes of rats fed menhaden oil. The β-adrenergic receptor concentration was not significantly altered although the affinity for [3H]dihydroalprenolol-binding was lower in membranes of rats fed menhaden oil than those fed the other oils. ω-3 fatty acids from menhaden oil were incorporated into the cardiac membrane phospholipids. The results suggest that the observed increase in myocardial adenylate cyclase activity of rats fed menhaden oil may be due to an increase in the number of the catalytic subunits of the enzyme or due to a greater availability of the forskolin-binding sites. ***Lipids 23,*** **207–213 (1988).**

The lipid dependence of adenylate cyclase has been shown by a number of studies (1–4). Because the composition of cell membrane lipids can be altered by dietary modifications, it is reasonable to expect concomitant changes in adenylate cyclase activity. Indeed, in several tissues changes in basal and hormone-stimulated adenylate cyclase activity have been observed after feeding diets containing different lipids (5–7). The changes in enzyme activity generally were associated with structural changes in membrane fatty acyl group composition. There is substantial evidence that the feeding of diets with different lipids results in modifying the fatty acid composition of cardiac phospholipids (8–10). There have been a number of studies to determine if the diet-induced changes in cardiac membranes also result in functional changes. Awad and Chattopadhyay (11) reported that feeding rats coconut oil-rich diet for four wk resulted in a significant decrease in 5′-nucleotidase, phosphodiesterase I and p-nitrophenylphosphatase activity of cardiac sarcolemma as compared with feeding rats safflower oil. In another study (12), the feeding of diets high in oleic, linoleic, linolenic and erucic acids to rats altered the fatty acid composition of mitochondrial phospholipids in rat heart, but did not affect the mitochondrial β-oxidation. In a previous study, we have shown that essential fatty acid (EFA) deficiency (13) resulted in a decrease in adenylate cyclase activity and β-adrenergic receptor content in rat heart. The changes in enzyme activity and in β-adrenergic receptor content were associated with modification in acyl group composition of cardiac membrane phospholipids. Because the fatty acid composition of cardiac membrane phospholipids can be altered by means other than an EFA deficiency such as by feeding oils of different fatty acid composition; this study was undertaken. Corn oil (relatively rich in ω-6 fatty acids), menhaden oil (relatively rich source of ω-3 fatty acids) and a mixture of 8% coconut oil + 2% corn oil (relatively rich in saturated fatty acids and sufficient in EFA) were used for the purpose of inducing structural changes in fatty acid composition of cardiac membrane phospholipids and to study their effects on adenylate cyclase system.

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Abbreviations: DHA, dihydroalprenolol; EFA, essential fatty acid.

MATERIALS AND METHODS

All the biochemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Dietary ingredients were purchased from Teklad (Madison, WI) except menhaden oil was a product of ICN Nutritional Biochemicals (Cleveland, OH). Radioisotopes: α-[32P]ATP (specific activity 619 Ci/mmol) was purchased from ICN (Cleveland, OH), [3H]cyclic AMP (specific activity 55 Ci/mmol), [3H]dihydroalprenolol (specific activity 38 Ci/mmol) were purchased from Amersham (Arlington Heights, IL) and [3H]forskolin (specific activity 40 Ci/mmol) was purchased from Dupont, NEN (Boston, MA).

Male weanling Sprague-Dawley rats (Holtzman Co., Madison, WI) were fed Purina Rat chow for seven days in order to get them acclimatized to the laboratory conditions. Rats were weighed and randomly divided into three groups of 27 rats each so that the average weight per group was the same. They were fed semipurified diets containing 10% corn oil (group 1), 8% coconut oil + 2% corn oil (group 2), or 10% menhaden oil (group 3). The diets contained (%): casein, 20.0; DL-methionine, 0.3; sucrose, 45.0; starch, 15.0; cellulose, 5.0; oil, 10.0; vitamin mixture (AIN-76A), 1.0; salt mixture (AIN-76A), 3.5; choline, 0.2; and BHT, 0.002. The diets were analyzed for their fatty acid composition after extracting 1 g aliquots of the freshly prepared diets with chloroform/methanol (2:1), esterifying aliquots of the lipid extracts with boron trifluoride methanol and subsequent gas chromatography on a Silar-10 C column as described (14).

The fatty acid composition of the three diets is shown in Table 1. The diet containing 10% corn oil had high levels of ω-6 fatty acids (58% of 18:2ω-6) and almost negligible amounts of ω-3 fatty acids. On the other hand, the diet containing menhaden oil had relatively high levels of ω-3 fatty acids (20:5, 22:5 and 22:6) and very low levels of ω-6 fatty acids. The diet supplemented with 8% coconut + 2% corn oil had high levels of medium chain saturated fatty acids, no ω-3 fatty acids and had some ω-6 fatty acids.

Rats were housed individually in suspended stainless steel cages in a temperature- and light-controlled room (22–23 C, 12 hr light-dark cycle) and were fed diets and tap water ad libitum for six wk. Although we did not measure the peroxide values of the diets, to minimize rancidity, the diets were stored at 4 C, and the diets left over in the food cups were discarded and replaced with the fresh diets daily. Rats were weighed at weekly intervals.

TABLE 1

Fatty Acid Composition of the Diets

Fatty acid	10% Corn oil	8% Coconut oil + 2% corn oil	10% Menhaden oil
12:0		34.8	
14:0		18.1	10.3
16:0	11.3	12.3	20.6
16:1	—	—	12.5
18:0	2.1	3.5	2.5
18:1	27.1	14.5	14.6
18:2 ω-6	58.4	16.9	1.0
18:3 ω-3	0.9	—	1.3
20:4 ω-6			0.5
20:5 ω-3			15.5
22:4 ω-6			1.1
22:5 ω-3			2.2
22:6 ω-3			8.6
Others	—	—	6.8

Values are in area percentage, average of duplicate analyses.
(—), Not detected.

At the termination of the experiment, rats were killed over a period of three days (between 8–10 a.m.). Hearts were removed, rinsed several times with cold physiological saline, chopped into small pieces with scissors and homogenized in a Potter-Elvejhem homogenizer with 9–10 volumes of a medium containing 0.32 M sucrose, 0.05 M Tris buffer (pH 7.4) and 1 mM $MgCl_2$. The homogenates were filtered through four layers of cheesecloth, and crude plasma membranes were prepared by differential centrifugation as described (13). Membranes and homogenates were aliquoted in small vials and stored in liquid nitrogen until assayed. No changes in adenylate cyclase activity or receptor binding were observed in samples stored in liquid nitrogen up to four wk. Aliquots of the homogenates and membranes were used for adenylate cyclase assays. The binding assays using [^{3}H]forskolin and [^{3}H]dihydroalprenolol (DHA) were carried out with the membranes.

β-receptor binding assays using [^{3}H]DHA as a radioligand were performed as described (13). Briefly, aliquots of cardiac membranes (150–350 μg protein) were incubated for 15 min with 0 to 29 nM [^{3}H]DHA in a final volume of 500 μl of a buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4 at 37 C). The bound radioligand was separated by vacuum filtration on Whatman GF/A glass fiber filter, and the radioactivity was determined by liquid scintillation. The specific binding of [^{3}H]DHA is defined as the difference between the total binding and the binding in the presence of 1 mM alprenolol. All assays were done in duplicates. The dissociation constant (K_d) and the concentration of the binding sites (B_{max}) were determined by Scatchard analysis using a computer.

Adenylate cyclase activity was measured in triplicate samples in homogenates and membranes by determining the conversion of [α-^{32}P]ATP to cyclic [^{32}P]AMP and isolating the product by sequential column chromatography (15). Details of the adenylate cyclase assay have been provided (14). The basal activity was measured without the addition of any stimulating agent in the incubation medium. The stimulated activity was measured in the presence of sodium fluoride (15 mM), isoproterenol (20 μM) or forskolin (0.1 M). In terms of the well-established three component adenylate cyclase system, isopreterenol activates the enzyme by stimulating the β-adrenergic receptor, fluoride by activating the guanine-binding regulatory proteins, and forskolin activates it by binding to the catalytic unit of the enzyme.

For [^{3}H]forskolin binding studies, cardiac membranes (25 μg protein) were incubated at 37 C for 30 min with 12.5 to 210 nM [^{3}H]forskolin in a final volume of 200 μl of buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4). Preliminary studies had shown that maximum binding of [^{3}H]forskolin was obtained under these conditions after 20 min of incubation and that there was no change in binding for up to 90 min. The specific binding was defined as the difference between the total binding and binding in the presence of 50 μM unlabeled forskolin. The unlabeled forskolin was dissolved in a mixture of ethanol/dimethyl sulfoxide (29:18%) in the above buffer as described by Ho and Shi (16). The final solvent concentrations for ethanol and dimethyl sulfoxide were 2.9 and 1.8%, respectively. At these concentrations, the solvents had little effect on radioligand binding or adenylate cyclase activity. At the end of the incubation period, all the tubes were placed in an ice-cold water bath followed by rapid filtration under vacuum through Whatman GF/A glass fiber filters. Filters were rapidly washed three times with 4 ml of the ice-cold incubation buffer to separate the bound and free radioligand. After drying, the bound radioactivity remaining on the filters was counted using a liquid scintillation spectrometer (Beckman LS6800). All binding assays were done in duplicate. The data for the saturation curves were analyzed by a computer to estimate the equilibrium dissociation constants (K_D) and the maximum number of the binding sites (B_{max}) using Scatchard analysis (17).

Fluorescence polarization was measured according to the procedures of Shinitzky and Inbar (18) and Shinitzky and Barenholz (19) as described (20). In brief, cardiac membranes were incubated with diphenyl hexatriene (DPH) in phosphate-buffered saline, and fluorescence polarization was measured using an SLM 4800 fluorescence polarization spectrophotometer (SLM Instruments, Inc., Urbana, IL) with two cross-polarized channels. The excitation was 366 nm. Scattering of the emitted light was reduced by using a 389 nm cut-off filter.

Total lipids were extracted from the cardiac homogenates and membranes using Bligh and Dyer's method (21). Phospholipids were separated by column chromatography (22). Methyl esters were prepared from aliquots of total lipids and phospholipids using boron trifluoride-methanol (23), and the fatty acid composition was determined by gas chromatography as described (14).

The data pertaining to the three dietary groups were treated statistically using analysis of variance and the significance of the differences among the groups was calculated using Newman-Keul's test (24).

RESULTS

There was no significant difference in the body weight gains of rats fed the various diets.

5′-Nucleotidase generally is used as a marker enzyme for plasma membranes. The crude membrane preparations that we used had 10–13-fold enrichment in 5′-nucleotidase activity over that of the homogenates. There was no significant difference in the relative purity of the membranes among the three dietary groups as evaluated by the activity of succinic dehydrogenase for mitochondria and glucose-6-phosphatase for microsomal contamination. Also, there was no difference in the yield of membranes among the three dietary groups.

Adenylate cyclase activity in heart homogenates (Table 2) was higher in rats fed menhaden oil than in the other two groups. The basal activity was 40–50% higher, and the stimulated activity (fluoride, isoproterenol or forskolin) was two- to three-fold higher in cardiac homogenates of rats fed diet containing menhaden oil than in that of the other two groups. There was essentially no difference in adenylate cyclase activity between the groups fed 10% corn oil or 8% coconut oil + 2% corn oil.

Adenylate cyclase activity in cardiac membranes of rats fed the three diets is shown in Table 3. As in the homogenates, basal and forskolin-stimulated activity was the highest of the three groups in membranes of rats fed diet containing menhaden oil. However, such differences were not observed in fluoride- or isoproterenol-stimulated activity. Although these activities still were higher in membranes of rats fed menhaden oil than those fed 8% coconut oil + 2% corn oil, there was no difference between the groups fed menhaden oil vs corn oil.

The saturation curves for the specific [^{3}H]DHA-binding to the cardiac membranes of rats fed diets containing different oils are shown in Figure 1A. The Scatchard plots (Fig. 1B) and the data in Table 4 show a lower affinity (higher K_D values) in membranes of rats fed menhaden oil than in the other two oils. There was a slight but nonsignificant ($P > 0.05$) increase in the number of binding sites in cardiac membranes of rats fed diet containing menhaden oil than the other two oils.

To determine the characteristics of [^{3}H]forskolin-binding to rat myocardial membranes, we conducted a preliminary study. Membranes were prepared from heart of adult male Sprague-Dawley rats by the differential centrifugation method similar to the one described above for isolating the cardiac membranes from rats fed the three diets.

The total, specific and nonspecific [^{3}H]forskolin binding as a function of the incubation time is shown in Figure 2. The binding was very rapid during-the first one to two min and reached a plateau at about 20 min of incubation. Therefore, in subsequent experiments a 30-min incubation time at 37 C was used routinely. The specific binding was linear over a range of 12.5 to at least 50 μg protein. Therefore, in subsequent experiments 50 μg or lower amount of membrane protein was used for [^{3}H]forskolin-binding studies.

The specific binding of [^{3}H]forskolin to the cardiac membranes of rats fed menhaden oil was higher than in those fed the other two oils. The values shown in Table 5,

TABLE 2

Adenylate Cyclase Activity in Heart Homogenates of Rats Fed Diets Containing Different Oils

Diet fed	Basal	+ Fluoride	+ Isoproterenol	+ Forskolin
10% Corn oil	13.7 ± 0.3^a	51.0 ± 10.3^a	31.0 ± 6.2^a	138.5 ± 18.5^a
8% Coconut oil + 2% corn oil	13.0 ± 0^a	52.8 ± 5.2^a	25.3 ± 4.3^a	137.8 ± 19.7^a
10% Menhaden oil	19.0 ± 0^b	110.5 ± 10.9^b	75.3 ± 11.8^b	291.5 ± 36.2^b

Values are mean ± SE of four assays/group; each assay was done in triplicate. Values with different superscripts in the same column are significantly different ($P < 0.01$) from each other using analysis of variance, Newman-Keul's test. Enzyme activity is shown in pmol/mg protein/min. The final concentrations of the activators in the incubation mixture were fluoride, 15 mM; isoproterenol, 20 μM; and forskolin, 0.1 mM.

TABLE 3

Adenylate Cyclase Activity in Heart Membranes of Rats Fed Diets Containing Different Oils

Diet fed	Basal	+ Fluoride	+ Isoproterenol	+ Forskolin
10% Corn oil	24.1 ± 1.1^a	163.2 ± 8.0^a	39.2 ± 2.4^a	328.7 ± 12.0^a
8% Coconut oil + 2% corn oil	21.0 ± 1.0^b	119.5 ± 4.2^b	28.3 ± 1.2^b	244.8 ± 20.4^b
10% Menhaden oil	27.9 ± 1.1^c	167.0 ± 6.6^a	43.0 ± 1.8^a	394.7 ± 13.5^c

Values are mean ± SE of three assays/group; each assay was done in triplicate. Values with different superscripts in the same column are significantly different ($P < 0.05$) from each other using analysis of variance, Newman-Keul's test. Enzyme activity is shown in pmol cAMP/mg protein/min. The final concentrations of the activators were fluoride = 15 mM, isoproterenol = 20 μM, and forskolin = 0.1 mM.

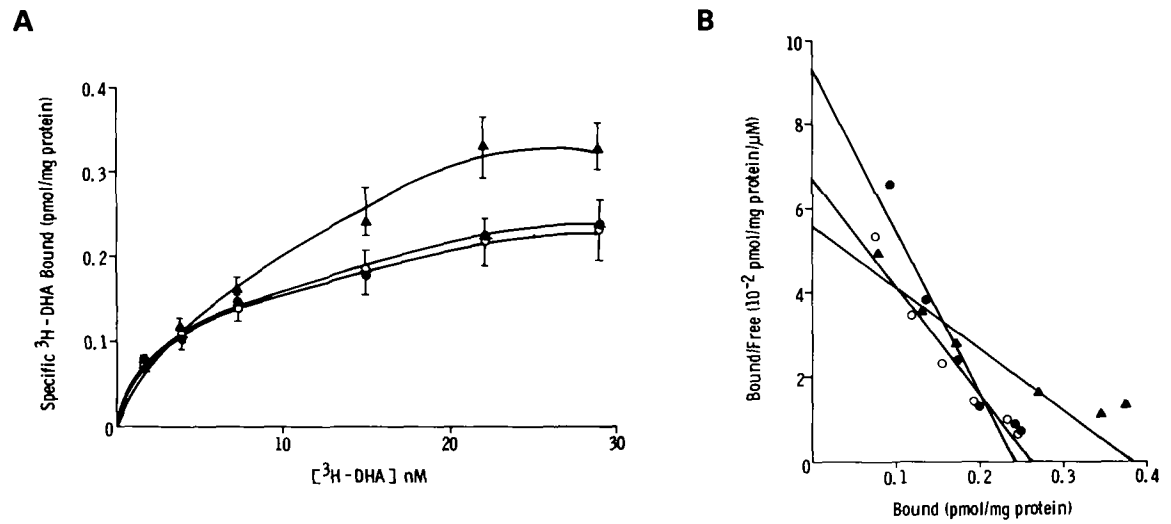

FIG. 1. (A) Saturation curves for the specific binding of [^{3}H]DHA to cardiac membranes of rats fed diets containing different oils. 10% corn oil, ●—●; 8% coconut oil + 2% corn oil, ○—○; 10% menhaden oil, ▲—▲. Each point represents a mean ± SE of four experiments, each done in duplicate. (B) Scatchard plot.

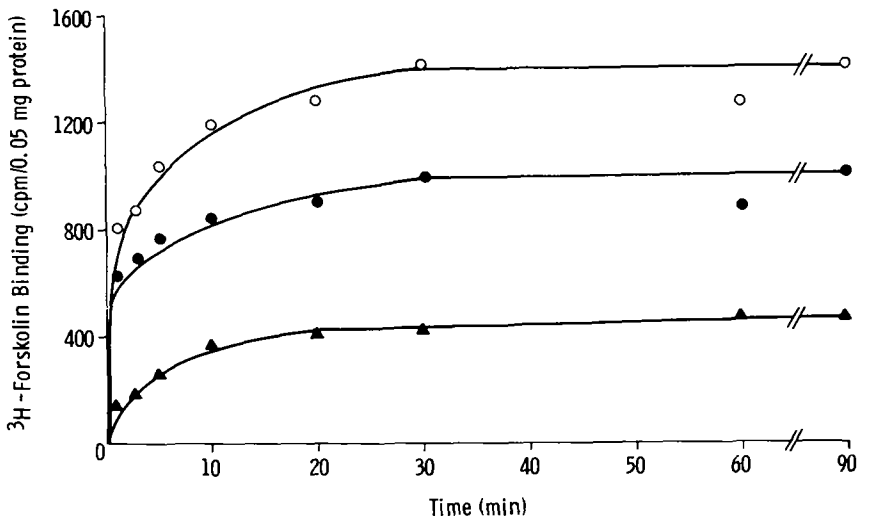

FIG. 2. Time course of [^{3}H]forskolin binding to cardiac membranes of rats. Membranes (50 μg protein) were incubated in 10 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4) and 50 nM [^{3}H]forskolin (final concentration) at 37 C for the specific time periods. Bound radioactivity was separated from the free radioactivity by rapid filteration assay as described in Methods. Each data point represents a mean of duplicate assays. Total binding, ○—○; nonspecific binding (in the presence of 50 μM forskolin), ▲—▲; specific binding, which represents the difference between the total binding and the nonspecific binding, ●—●.

TABLE 4

[^{3}H]DHA-Binding to Cardiac Membranes of Rats Fed Diets Containing Different Oils

Diet fed	K_D	B_{max}
	nM	pmol/mg protein
10% Corn oil	2.44 ± 0.41[a]	0.223 ± 0.041[a]
8% Coconut oil + 2% corn oil	3.50 ± 0.48[a]	0.234 ± 0.047[a]
10% Menhaden oil	6.54 ± 1.16[b]	0.357 ± 0.076[a]

Values are mean ± SE of four assays/group; each was done in duplicate. Values with different superscripts in the same column are significantly different P(<0.05) from each other using analysis of variance, Newman-Keul's test.

TABLE 5

[^{3}H]Forskolin-Binding to Rat Heart Membranes

Diet fed	K_D	B_{max}
	μM	pmol/mg protein
10% Corn oil	0.24 ± 0.03[a]	4.75 ± 0.16[a]
8% Coconut oil + 2% corn oil	0.22 ± 0.04[a]	4.49 ± 0.67[a]
10% Menhaden oil	0.27 ± 0.04[a]	8.42 ± 0.52[b]

Values are mean ± SE of three assays/group; each was done in duplicate. Values with different superscripts in the same column are significantly different P(<0.01) from each other using analysis of variance, Newman-Keul's test.

which were calculated from the Scatchard plots, show a higher concentration of the binding sites in membranes of rats fed menhaden oil than in the other two groups. There was, however, no difference in the affinity of the binding sites among the three groups.

The stimulation in adenylate cyclase activity with different concentrations of forskolin (Fig. 3) also showed a significant increase in the enzyme activity in cardiac membranes of rats fed diets containing menhaden oil. This was the case at each concentration of forskolin.

The fatty acid composition of total phospholipids of cardiac homogenates and membranes (Table 6) showed relatively higher proportions of 22:6 ω-3 (17–18% of the total fatty acids) and 20:5 ω-3 (4–5% of the total fatty acids) in rats fed menhaden oil than the other two oils. The levels of ω-6 fatty acids (18:2, 20:4 and 22:4) were lower in rats fed diet containing menhaden oil than those in the other two dietary groups. The double bond index, a measure of the degree of unsaturation, was higher in membrane phospholipids of rats fed diet containing menhaden oil than in the other two oils (173 in 10% corn oil, 171 in 8% coconut oil + 2% corn oil, and 213 in the group fed 10% menhaden oil).

The fluorescence polarization of DPH measured at different temperatures in cardiac membranes was essentially the same among the three dietary groups (Fig. 4).

DISCUSSION

We have reported (13) that EFA deficiency resulted in a decrease in adenylate cyclase activity and in the concentration of β-adrenergic receptor in rat heart. Since the levels of linoleic acid in menhaden oil are quite low, this raises the possibility of an EFA deficiency by feeding of a diet containing 10% menhaden oil. However, there was no evidence of an EFA deficiency as shown by the fatty acid composition of total lipids of plasma (data not shown). Although the 20:4ω-6 levels were lower in rats fed 10% menhaden oil (10% vs 32–33% in the other two dietary groups), there was no accumulation of 20:3ω-9. It constituted less than 0.5% of the total fatty acids in plasma of rats fed 10% menhaden oil.

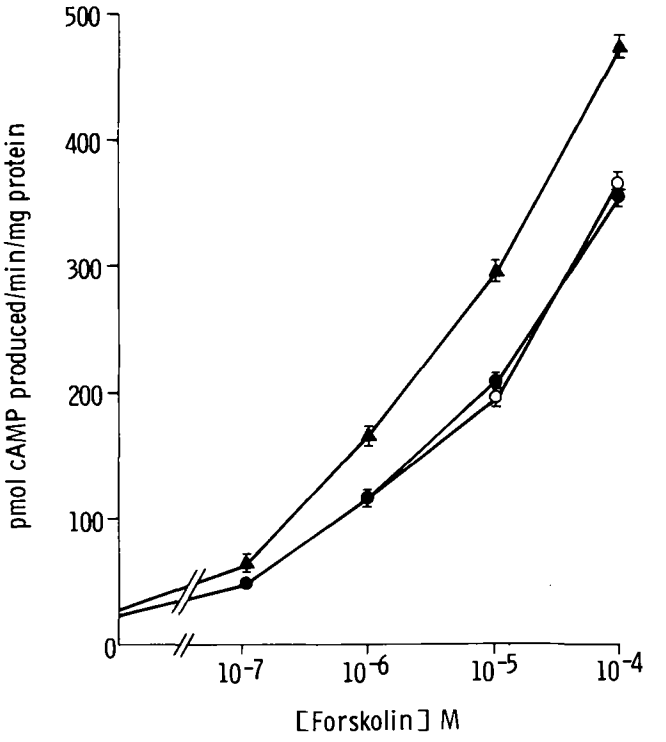

FIG. 3. Forskolin-stimulated adenylate cyclase activity in cardiac membranes of rats fed diets containing different oils. 10% corn oil, ●—●; 8% coconut oil + 2% corn oil, ○—○; 10% menhaden oil, ▲—▲. Each point represents a mean of four experiments, each done in triplicate. The bars represent SE.

TABLE 6

Fatty Acid Composition of Total Phospholipids of Heart Homogenates and Membranes of Rats Fed Diets Containing Different Oils

Fatty acid	10% Corn oil		8% Coconut oil + 2% corn oil		10% Menhaden oil	
	Homogenate	Membrane	Homogenate	Membrane	Homogenate	Membrane
14:0	1.0 ± 0.1[a]	1.6 ± 0.2[a]	1.5 ± 0.2[a]	1.6 ± 0.1[a]	1.6 ± 0.2[a]	1.6 ± 0.2[a]
16:0	12.9 ± 0.8[a]	14.0 ± 0.5[a]	13.7 ± 0.9[a]	14.2 ± 0.6[a]	14.8 ± 0.9[a]	15.9 ± 0.6[a]
16:1	0.6 ± 0.1[a]	0.9 ± 0.3[a]	1.1 ± 0.1[b]	0.9 ± 0.1[a]	3.0 ± 0.2[c]	2.6 ± 0.1[b]
18:0	23.2 ± 0.4[a]	24.6 ± 0.2[a]	22.8 ± 0.6[a]	22.6 ± 0.1[a]	23.1 ± 0.6[a]	24.9 ± 0.1[a]
18:1	8.3 ± 0.9[a]	8.4 ± 0.5[a]	11.8 ± 1.0[a]	11.4 ± 0.2[b]	11.2 ± 0.9[a]	11.1 ± 0.1[b]
18:2 ω-6	21.1 ± 0.1[a]	16.5 ± 0.2[a]	19.5 ± 0.5[b]	16.3 ± 0.3[a]	7.1 ± 0.3[c]	6.4 ± 0.1[b]
20:4 ω-6	21.2 ± 1.2[a]	21.3 ± 1.0[a]	21.5 ± 0.1[a]	21.5 ± 0.5[a]	11.1 ± 0.3[b]	11.1 ± 0.3[b]
20:5 ω-3	—[a]	—[a]	—[a]	—[a]	5.0 ± 0.2[b]	4.7 ± 0.3[b]
22:4 ω-6	4.1 ± 0.3[a]	4.2 ± 0.3[a]	4.2 ± 0.2[a]	4.3 ± 0.3[a]	0.3 ± 0.1[b]	0.3 ± 0.1[b]
22:5 ω-3	0.3 ± 0.1[a]	0.5 ± 0.1[a]	—[b]	—[b]	3.0 ± 0.2[c]	3.1 ± 0.3[c]
22:6 ω-3	4.9 ± 0.1[a]	4.3 ± 0.5[a]	3.7 ± 0.5[a]	3.8 ± 0.7[a]	18.1 ± 0.9[b]	16.9 ± 0.3[b]

Values are in area percentage, averages of triplicate analyses within each group (mean ± SEM).

—, Not detected. Values (for homogenates or membrane) with different superscript letters in the same row are significantly different ($P < 0.01$) using analysis of variance, Newman-Keul's test.

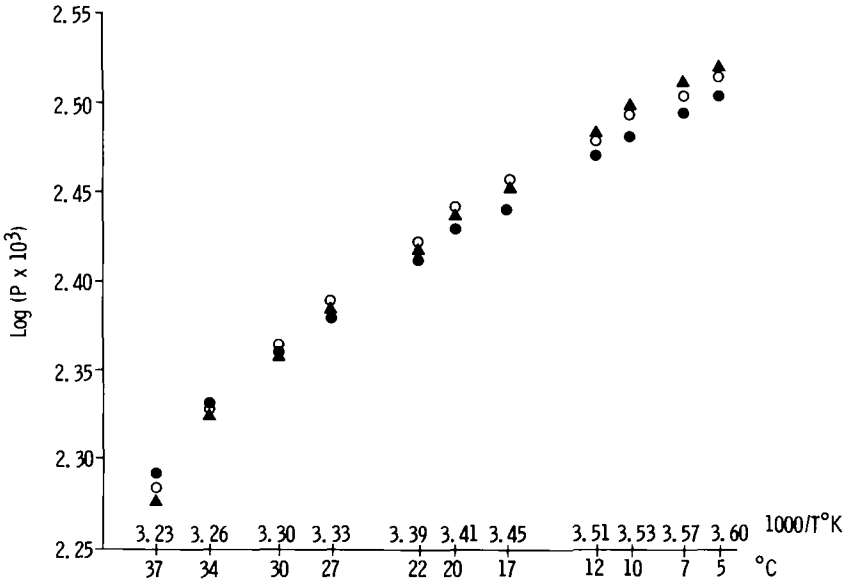

FIG. 4. Fluorescence polarization of DPH in cardiac membranes of rats fed diets containing 10% corn oil, ●; 8% coconut oil + 2% corn oil, ○; or 10% menhaden oil, ▲. Fluorescence polarization was measured at different temperatures (5–37 C).

Adenylate cyclase system consists of a hormone-sensitive receptor (R), guanine-nucleotide binding regulatory proteins (G_s and G_i) and the catalytic subunit (C). Changes in any of these components theoretically may explain the increase that we observed in the stimulated adenylate cyclase activity in heart of rats fed menhaden oil. Since the B_{max} of [^{3}H]forskolin binding was almost doubled in cardiac membranes of rats fed diet containing menhaden oil, it appears that the main reason for an increase in adenylate cyclase activity in this group is an increase in the number of the catalytic subunits of adenylate cyclase or a greater availability of the [^{3}H]-forskolin-binding sites. The catalytic subunit being the last step in the adenylate cyclase activation very well could become the rate-limiting step. Therefore, any increase in the concentration of this catalytic subunit by feeding menhaden oil also would result in a higher adenylate cyclase activity, even if the β-adrenergic receptor concentration and that of the G proteins remains the same.

Cardiac membrane phospholipids showed a preponderance of 20:5, 22:5 and 22:6 in rats fed menhaden oil; the level of 22:6 were especially high (17% of total). This was at the expense of ω-6 fatty acids such as 18:2, 20:4 and 22:4, which were reduced. Since the highly unsaturated fatty acids such as 20:5, 22:5 and 22:6 would constitute a more fluid domain around the catalytic subunit, it is tempting to speculate that a greater number of the binding sites would be available for [^{3}H]forskolin as was observed in membranes of this group.

In addition to the fatty acid composition of the membrane phospholipids, other variables such as cholesterol to phospholipid ratio and the proportion of various phospholipids also could influence membrane fluidity. Although we did not measure these parameters, the fluidity of the membranes among the three dietary groups was essentially the same as measured by fluorescence polarization of DPH. Since fluorescence polarization measures the "average" fluidity of the membrane, this observation does not exclude changes in the enzyme microenvironment.

[^{3}H]forskolin binding sites characteristics have been examined in heart of rats fed a standard diet (25). Our data on B_{max} and K_D for forskolin binding are consistent with this finding. In another study (26), the effect of feeding coconut oil (rich in saturated fatty acids) and sunflower oil (rich in polyunsaturated fatty acids) on rat heart adenylate cyclase and β-adrenergic receptor was examined. A decrease in adenylate cyclase activity (basal, norepinephrine- and fluoride-stimulated) was reported in atrial homogenates of rats fed sunflower oil relative to the coconut oil. The K_D and B_{max} of [^{3}H]DHA-binding also were decreased in atrial homogenates from rats fed sunflower oil. The values for the affinity and the concentration of [^{3}H]DHA-binding sites in our study essentially are similar to those obtained by Wince and Rutledge (26). Although the incorporation of fish oil fatty acids into cardiac (27) and other tissues (28) has been reported, to our knowledge, there are no previous studies on the effects of fish oils on cardiac adenylate cyclase system or on [^{3}H]forskolin-binding sites in heart as affected by dietary lipids.

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REFERENCES

1. Lefkowitz, R.J. (1975) *J. Mol. Cell. Cardiol. 7*, 27–37.
2. Levey, G.S. (1971) *J. Biol. Chem. 246*, 7406–7410.
3. Limbird, L.E., and Lefkowitz, R.J. (1976) *Mol. Pharmacol. 12*, 559–567.
4. Sutherland, E.W., Rall, T.W., and Menon, T. (1962) *J. Biol. Chem. 237*, 1220–1227.
5. Morson, L.A., and Clandinin, M.T. (1986) *J. Nutr. 116*, 2355–2362.

6. Neelands, P.J., and Clandinin, M.T. (1983) *Biochem. J. 212,* 573-583.
7. Yamaguchi, K., and Matsuoka, A. (1982) *Horm. Metabol. Res. 14,* 117-121.
8. Charnok, J.S., Abeywardena, M.Y., McMurchie, E.J., and Russell, G.R. (1984) *Lipids 19,* 206-213.
9. Gudbjarnason, S., and Oskardottir, G. (1977) *Biochim. Biophys. Acta 487,* 10-15.
10. Tahin, Q.S., Blum, M., and Carafoll, E. (1981) *Eur. J. Biochem. 121,* 5-13.
11. Awad, A.B., and Chattopadhyay, J.P. (1983) *J. Nutr. 113,* 1878-1884.
12. Lee, S-H.C., and Clandinin, M.T. (1986) *J. Nutr. 116,* 2096-2105.
13. Alam, S.Q., Alam, B.S., and Ren, Y-F. (1987) *J. Mol. Cell. Cardiol. 19,* 465-475.
14. Alam, S.Q., and Alam, B.S. (1986) *J. Nutr. 116,* 1620-1630.
15. Salomon, Y., Londos, C., and Rodbell, M. (1974) *Anal. Biochem. 58,* 541-548.
16. Ho, R-J., and Shi, Q-H. (1984) *J. Biol. Chem. 259,* 7630-7636.
17. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51,* 660-672.
18. Shinitzky, M., and Inbar, M. (1974) *J. Mol. Biol. 85,* 603-615.
19. Shinitzky, M., and Barenholz, Y. (1974) *J. Biol. Chem. 249,* 2652-2657.
20. Alam, S.Q., Alam, B.S., and Banerji, A. (1985) *Lipid 20,* 16-23.
21. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
22. Rouser, G., Kritchevsky, G., and Yamamoto, A. (1967) in *Lipid Chromatographic Analysis* (Marinetti, G.V., ed.), Vol. 1, pp. 118-120, Dekker, New York, NY.
23. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600-608.
24. Snedecor, G.W., and Cochran, W.G. (1967) in *Statistical Methods,* 6th edn., pp. 273-275, The Iowa State University Press, Ames, IA.
25. Schmidt, K., and Kukovetz, W.R. (1985) *J. Cyclic Nucleotide Protein Phosphor. Res. 10,* 425-438.
26. Wince, L.C., and Rutledge, C.O. (1981) *J. Pharmacol. Exp. Ther. 219,* 625-631.
27. Swanson, J.E., and Kinsella, J.E. (1986) *J. Nutr. 116,* 514-523.
28. Dyerberg, J., Bang, H.O., Moncada, S., and Vane, J.R. (1978) *Lancet 2* (8081), 117-119.

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Structural Modifications of Rat Serum High Density Lipoprotein by Pancreatic Phospholipase A_2

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An important and unusual aspect of the high density lipoprotein (HDL) in the rat is its tendency to undergo marked alterations in structure in response to physiological perturbations. In this study, the role of the surface lipids for maintenance of HDL integrity were investigated. Hydrolysis by pancreatic phospholipase A_2 of the phospholipids of rat HDL in the presence of the d > 1.21 g/ml fraction of rat serum results in an increase in the particle diameter and an uptake of apo-E and apo A-IV from the lipoprotein-free fraction; augmentation of the albumin concentration in the incubation mixture intensified the observed changes, probably due to enhancement of the compositional changes brought about by phospholipase treatment. Phospholipase A_2 treatment of the d < 1.21 g/ml fraction of rat serum produces only minor changes in the properties of the isolated HDL. These data suggest that changes in apoprotein content reflect an uptake of A-IV and E by the rat HDL, rather than a net loss of apo A-I. Likewise, titration of the action of pancreatic phospholipase A_2 on HDL apoprotein composition showed that initially a modest increase in apo A-IV content occurred, but with more extensive phospholipolysis there was a considerably greater increase in the apo-E content of the particle. The data suggest that hydrolysis of phospholipids such as occurs physiologically through the action of lecithin:cholesterol acyl transferase and hepatic lipase may alter the HDL structure independently from changes effected in the neutral lipid core.
Lipids 23, 214–219 (1988).

An important feature of the plasma lipoproteins is that these complex protein-lipid assemblies undergo continuous remodeling in the circulation due to the action of enzymes involved in lipid metabolism, such as lecithin:cholesterol acyl transferase (LCAT) and hepatic lipase. Proteins responsible for the transfer of both neutral and polar lipid components among the lipoprotein classes appear to influence HDL structure as well (1). An important consequence of the structural rearrangements effected by these agents is the observed heterogeneity within lipoprotein classes.

An intriguing feature of lipid metabolism in the rat is the tendency for rat HDL to show marked changes in apoprotein distribution in response to various metabolic stimuli. For example, feeding a diet high in cholesterol causes rat HDL to become depleted in apo-E (2,3), while induction of diabetes causes a loss of both apo-E and apo A-IV (2,4). By contrast, the apoprotein content of human HDL has been found to be fairly constant.

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Abbreviations: BSA, bovine serum albumin; CE, cholesterol ester; CHOL, cholesterol; DTNB, 5,5′-dithiobis(2-nitrobenzoate); FFA, free fatty acid; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyl transferase; LDL, low density lipoprotein; LYSO, lysophosphatidylcholine; PL, phospholipid; PLASE, phosphalipase A_2; SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate; TG, triglyceride; VLDL, very low density lipoprotein.

Recent investigations have documented that the LCAT enzyme causes major modifications of the HDL molecule, which are reflected in altered chemical composition and increased size (5–7). In the rat, there has been considerable evidence that there also are major redistributions of apoproteins resulting from LCAT action, with increased amounts of apo A-IV and apo-E becoming associated with the particle (7,8). Since the first step in the LCAT-mediated reaction is a phospholipase A_2-like reaction, we initiated studies to evaluate whether phospholipase A_2 alone would cause the changes in HDL properties associated with LCAT, apart from the cholesteryl ester formation catalyzed by LCAT.

Earlier studies on the treatment of isolated human HDL by snake venom phospholipases showed that despite extensive hydrolysis of fatty acids from phosphatidylcholine and phosphatidylethanolamine, the HDL remained intact and retained its physical properties (9,10). Preliminary studies in our laboratories showed that phospholipase treatment of rat serum followed by isolation of the HDL fraction resulted in considerable change to both the chemical and physical properties of the HDL. The present studies were undertaken to compare the action of phospholipase on HDL in the presence or absence of serum components and to provide more complete chemical characterization of these products than has been available previously.

MATERIALS AND METHODS

Serum treatment and HDL isolation. Serum was obtained from male Sprague-Dawley rats (270–350 g) by exsanguination from the abdominal aorta. In all cases, 5-5′-dithiobis(2-nitrobenzoate) (DTNB) in 0.075 M sodium phosphate buffer, pH 7.4, was added immediately upon collection (final concentration, 1.5 mM) to inhibit LCAT activity. All blood samples were allowed to clot on ice for three hr, and the serum was recovered by centrifugation. For some studies, various fractions, including the d > 1.21, the d < 1.21, and the d 1.063–1.21 g/ml fraction, were isolated by ultra-centrifugation (11). After dialysis against 0.15 M NaCl containing 0.01% EDTA pH 7.4, the various fractions or whole serum were incubated with porcine pancreatic phospholipase A_2 (Sigma Chemical Co., St. Louis, MO) for 18 hr at 37 C. In most studies, the amount of phospholipase used was 15 units/ml; calcium chloride was added to the incubations at final concentrations of either 3 mM or 21 mM. Incubations of HDL or the d < 1.21 fraction were buffered with 0.2 M Tris-HCl, pH 7.5, to prevent precipitation due to changes in pH during lipolysis.

In some cases, albumin was added incrementally to the mixture; this was added either before the incubation or afterward, followed by a 6–18 hr equilibration at 4 C to allow binding of the lipolytic products.

The HDL fraction was isolated in the d 1.063–1.21 density interval by the addition of KBr to the incubation mixture and ultracentrifugation. This material was

deliberately not "washed" at d 1.21 g/ml in order to minimize losses that are known to result from ultracentrifugation. The d < 1.063 material was dialyzed against 0.15 m NaCl, 0.27 mM EDTA, pH 7.5, and centrifuged for 20 hr to obtain the VLDL. All fractions were dialyzed against 0.15 M NaCl, 0.27 mM EDTA, pH 7.6, before analysis.

Titration of phospholipase action on apoprotein distribution. To evaluate the effects of phospholipase at various concentrations, rat serum was incubated at 37 C for 18 hr with various concentrations of phospholipase A_2 ranging from .005–15 units/ml. After incubation, the HDL was isolated by ultracentrifugation and analyzed by SDS gel electrophoresis; relative apoprotein quantitation was achieved by scanning densitometry of the stained gels.

Pore limit electrophoresis. The size of HDL particles was determined using a 4–30% acrylamide gradient gel essentially as described by Anderson et al. (12) using a voltage of 150 V for 44 hr applied to Pharmacia PAA 4/30 gels (Piscataway, NJ) or, alternatively, gradient slab gels were prepared and run in a homemade apparatus. The sample volume was adjusted in each case so that 10 μg of protein were applied in each slot; following electrophoresis, the gel was stained with Coomassie Brilliant Blue G-250. Protein standards for estimating molecular dimensions were purchased from Pharmacia, and the following values for Stokes' diameter were used: thyroglobulin, 17.0 nm; apoferritin, 12.2 nm; catalase, 10.2 nm; lactate dehydrogenase, 8.1 nm; and serum albumin, 7.1 nm (13). The relationship between molecular size and electrophoretic mobility in these gels originally was described by Felgenhauer (14).

SDS gel electrophoresis and chemical cross-linking. SDS slab gel electrophoresis was performed using 3–27% acrylamide gels as described (15). Sample volumes were adjusted so that 25 μg of protein was loaded in each lane of the gel. Gels were stained with Coomassie Brilliant Blue G-250 and destained in 7% acetic acid, 5% methanol. In some cases, HDL samples were cross-linked using dimethylsuberimidate (Pierce Chemical Co., Rockford, IL) (16) and were electrophoresed on SDS gels along with cross-linked apo A-I and hemocyanin (Sigma Chemical Co.) and cross-linked bovine serum albumin as molecular weight standards.

Chemical analyses. Protein content was determined by the method of Lowry et al. (17) as modified by Markwell et al. (18). Total and free cholesterol were measured enzymatically, using a reagent kit prepared by Fermco (Elk Grove Village, IL); a sample of control human serum (Control H, Sigma Chemical Co.) was analyzed on each occasion to verify the reproducibility of results from day-to-day. The mass of cholesteryl ester was computed from the difference between total and free cholesterol by utilizing a factor of 1.7. Triglyceride was determined enzymatically using a kit prepared by Sigma Chemical Co. Free fatty acids were analyzed enzymatically using reagents (NEFA kit) prepared by Wako Chemical Company (Dallas, TX). Phospholipid phosphorus was quantitated by the method of Marinetti et al. (19). Phosphorus was corrected to mass of phospholipid by using a factor of 25 for intact phospholipid and 17 for lysophosphatidylcholine.

Phospholipid species were determined by analysis of the phosphorus in lipid spots separated by thin layer chromatography. Folch extracts were spotted on Silica Gel 60 plates (E. Merck, Darmstadt) and chromatographed using chloroform/methanol/acetic acid/water 25:14:4:2, v/v/v/v).

Statistical analysis. Data were analyzed for statistical significance using the Students' t-test.

RESULTS

Effect of phospholipolysis upon HDL composition. A summary of the effects of phospholipolysis on the chemical composition of isolated HDL are presented in Tables 1 and 2; data on the incubation of pancreatic phospholipase A_2 with whole serum or with the HDL fraction (d1.063–1.21 g/ml) plus the d > 1.21 g/ml fraction are given in Table 1, while data for incubation of phospholipase with the d < 1.21 g/ml fraction or with HDL is shown in Table 2.

The action of phospholipase on HDL in the presence of the d > 1.21 fraction (either as d > 1.21 or in whole serum) (Table 1) led to increases in protein content simultaneous with loss of intact phospholipid and accumulation of lysolecithin and free fatty acid. Specifically, the protein in HDL of whole serum increased from 40.7% to 48.8% ($p < .005$), and for HDL + d > 1.21 g/ml the protein content increased from 36% to 45% ($p < .005$). The addition of bovine serum albumin (BSA) either during the incubation or following the incubation led to a major reduction in lysolecithin and free fatty acid.

On the other hand, phospholipolysis of either HDL or the d < 1.21 g/ml fraction (Table 2) did not lead to significant increases in the protein content despite extensive hydrolysis of phospholipid (i.e., HDL protein content changed from 33.0% to 33.5% when HDL alone was treated with phospholipase, while this value changed from 37.4% to 40.7% for the d < 1.21 g/ml fraction). However, there was much greater accumulation of FFA and lysolecithin in the absence of the d > 1.21 components as compared with Table 1. The addition of albumin reduced the FFA and lysolecithin contents and increased the percentage of other lipoprotein constituents.

Effect of phospholipolysis on HDL particle size. The Stokes' diameter of the HDL particle following phospholipolysis under the conditions described for Tables 1 and 2 was determined by pore limit electrophoresis, and results are summarized in Table 3. Phospholipase A_2 treatment of whole serum or HDL plus the d > 1.21 fraction led to about a 1 nm increase in HDL diameter, while the presence of albumin yielded an increment of about 2 nm. On the other hand, phospholipolysis of HDL alone or of the d < 1.21 g/ml fraction led to a decrease of 0.4 nm; inclusion of albumin prevented this decrease in size but did not produce a significant increase in diameter.

Effect of phospholipolysis upon HDL protein content per particle. The utility of chemical cross-linking for establishing the relative amounts of protein on human HDL_2 and HDL_3 has been demonstrated previously (20). The total mass of protein located on the rat HDL particle isolated after the phospholipolysis treatments described above was estimated by cross-linking the particle with dimethylsuberimidate, followed by SDS gel electrophoresis to determine mass relative to cross-linked standards. One such experiment is shown in Figure 1; molecular weights were determined relative to cross-linked apo A-I, cross-linked bovine serum albumin, and

TABLE 1

Chemical Composition of HDL Reisolated from Whole Rat Serum or from an HDL + d > 1.21 g/ml Mixture Incubated in the Presence or Absence of Pancreatic Phospholipase[a]

No. of expts.[b]	Serum fraction	PLASE	BSA[c]	Weight percentage						
				Protein	TG	CHOL	CE	LYSO	Intact PL[d]	FFA
2	Whole serum	–	–	40.7	1.9	4.7	24.5	1.7	26.1	0.9
2	Whole serum	+	–	48.8	2.3	3.8	28.1	10.1	3.8	3.7
1	Whole serum	+	+	53.1	2.7	3.5	34.5	1.2	4.0	1.0
2	HDL + d > 1.21 g/ml	–	–	36.0	1.7	6.0	25.5	1.6	29.0	0.5
2	HDL + d > 1.21 g/ml	+	–	45.0	2.8	8.2	26.5	7.8	6.7	2.3
4	HDL + d > 1.21 g/ml	+	+	52.0	2.3	7.6	31.3	1.0	5.1	0.5

[a]Whole rat serum or HDL (d 1.063–1.21 g/ml) reconstituted with d > 1.21 g/ml was incubated at 37 C for 18 hr with or without pancreatic phospholipase A_2 (15 units/ml).

[b]Chemical composition data were obtained in each of the indicated number of experiments. Within each experiment, each determination was performed two separate times, each using duplicate samples.

[c]Bovine serum albumin was added to increase the albumin concentration by 50 mg/ml.

[d]All phospholipids except lysophosphatidylcholine.

TABLE 2

Chemical Composition of HDL Reisolated from d < 1.21 g/ml Fraction or HDL Treated with Phospholipase[a]

No. of expts.[b]	Serum fraction	PLASE	BSA[c]	Weight percentage						
				Protein	TG	CHOL	CE	LYSO	Intact PL[d]	FFA
2	d < 1.21 g/ml	–	–	37.4	1.5	4.7	22.6	4.2	26.7	2.6
2	d < 1.21 g/ml	+	–	40.7	1.5	4.1	22.9	15.4	2.9	12.2
1	d < 1.21 g/ml	+	+	47.0	2.6	4.4	26.0	11.7	4.3	3.8
2	HDL	–	–	33.0	3.7	5.5	24.2	3.3	29.1	0.8
2	HDL	+	–	33.5	3.8	6.1	25.8	15.7	7.1	8.6
4	HDL	+	+	51.0	3.3	7.5	31.3	1.7	4.4	0.7

[a]The d < 1.21 g/ml fraction or HDL (d 1.063–1.21 g/ml) were incubated at 37 C for 18 hr with or without pancreatic phospholipase A_2 (15 units/ml).

[b]Chemical composition data were obtained in each of the indicated number of experiments. Within each experiment, each determination was performed two separate times, each using duplicate samples.

[c]Bovine serum albumin was added to increment the albumin concentration by 50 mg/ml for HDL, but only by 17 mg/ml for the d < 1.21 g/ml sample.

[d]All phospholipids except lysophosphatidylcholine.

TABLE 3

Stokes' Diameter (from Pore Limit Electrophoresis) of HDL Reisolated from Phospholipase Serum Fractions[a]

Serum or d > 1.063	11.2 ± 0.2 (8)[b] [A][c]	12.1 ± 0.9 (8) [B]	13.2 ± 0.9 (7) [C]
d < 1.21 or HDL	10.7 ± 0.3 (7) [D]	10.3 ± 0.3 (7) [E]	10.8 ± 1.2 (5) [F]

[a]Stokes' diameters in nm for HDL reisolated from incubation mixtures of the indicated serum fractions treated at 37 C for 18 hr with or without pancreatic phospholipase A_2 and with or without BSA added to increment the albumin concentration by 50 mg/ml.

[b]Stokes diameter ± standard deviation. The number in parentheses indicates the number of separate determinations.

[c]The probabilities that the population means are the same for the following pairs of observations: [A] – [B], $p < .02$; [B] – [C], $p < .02$; [D] – [E], $p < .03$; [E] – [F], not significant.

TABLE 4

Protein Mass Per Particle of HDL Reisolated from Phospholipase Serum Fractions[a]

Sample	−PLASE	+PLASE	+PLASE,+BSA
Serum or d > 1.063	127 ± 7 (9)[b] [A][c]	158 ± 7 (9) [B]	222 ± 17 (3) [C]
d < 1.21 or HDL	110 ± 17 (6) [D]	110 ± 10 (6) [E]	137 ± 15 (3) [F]

[a]Protein mass in kilodaltons as determined by SDS gel electrophoresis of cross-linked HDL reisolated from incubation mixtures of the indicated serum fractions treated at 37 C for 18 hr with or without pancreatic phospholipase A_2, and with or without BSA added to increase the albumin concentration by 50 mg/ml.

[b]Protein mass ± standard deviation. The number in parentheses indicates the number of separate determinations.

[c]The probabilities that the population means are the same for the following pairs of observations: [A] − [B], $p < .001$; [B] − [C], $P < .03$; [D] − [E], not significant; [E] − [F], not significant.

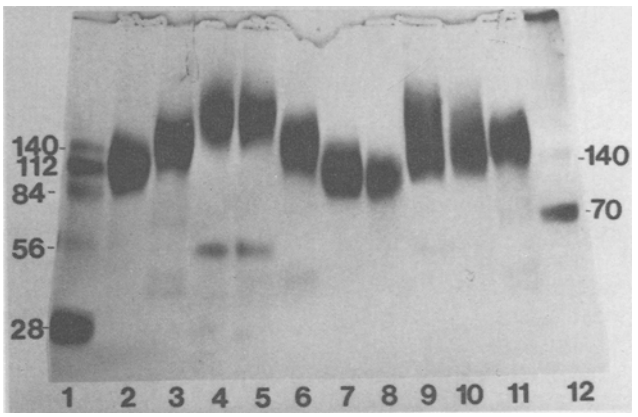

FIG. 1. Cross-linking of HDL isolated from incubation mixtures of isolated HDL with other serum fractions, in the presence or absence of phospholipase. Cross-linked apo A-I oligomers (Lane 1) and cross-linked hemocyanin oligomers (Lane 12) are run as markers for protein mass; molecular weights for these oligomers (in kilodaltons) are indicated on the sides of the figure. The incubations used for preparing the HDL run in lanes 2–11 all contained HDL (d 1.063–1.21 g/ml) and the following additions: Lane 2, d > 1.21; Lane 3, d > 1.21 and PLASE; Lane 4, d > 1.21, BSA, and PLASE; Lane 5, d > 1.21 and PLASE, with addition of BSA after incubation; Lane 6, d < 1.006 g/ml and PLASE; Lane 7, no additions; Lane 8, PLASE; Lane 9, BSA and PLASE; Lane 10, PLASE, with addition of BSA after incubation; Lane 11, d > 1.21 and PLASE (3 mM Ca^{++}).

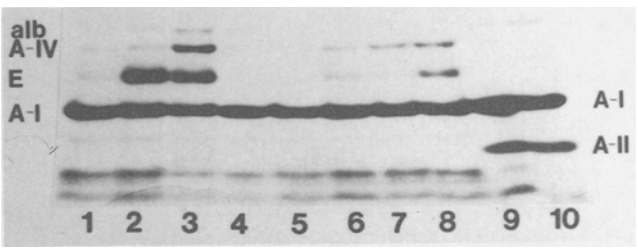

FIG. 2. SDS-PAGE of HDL isolated from incubation mixtures containing the following compositions: Lane 1, rat serum; Lane 2, rat serum plus PLASE; Lane 3, rat serum plus BSA plus PLASE; Lane 4, rat serum; Lane 5, rat HDL; Lane 6, rat HDL plus PLASE; Lane 7, rat d < 1.21 g/ml; Lane 8, rat d < 1.21 g/ml plus PLASE; Lane 9, human serum; Lane 10, human serum plus PLASE. All incubations contained 1.4 mM DTNB and 3 mM Ca^{++}.

cross-linked hemocyanin. Average values from several experiments are presented in Table 4. When whole serum or HDL plus the d > 1.21 g/ml fraction were treated with phospholipase, the HDL protein mass increased by about 30,000 and by almost 100,000 when albumin was included in the incubation or added afterward. On the other hand, phospholipolysis of isolated HDL or of the d < 1.21 g/ml fraction yielded no increase in protein mass unless albumin was present, and then only an increase of 25,000 was observed.

It should be noted that in cross-linked samples from incubations with added BSA, a band is observed at a molecular weight corresponding to the albumin monomer (Fig. 1, lanes 4 and 5). This reflects the fact that some albumin contamination of the isolated HDL occurs because of its high concentration but that most or all of it does not reside on the HDL particle itself.

Effect of phospholipolysis upon apoprotein distribution. The relative content of the HDL apoproteins was determined by SDS gel electrophoresis of the various HDL species studied. Figure 2 shows some typical results with HDL isolated from phospholipase-treated serum or HDL isolated from a d < 1.21 g/ml fraction treated with phospholipase. Although there was some between-sample variation, in general there was always a major increase in the proportions of apo E and apo A-IV, in serum or HDL + d > 1.21 samples following phospholipolysis either in the presence or absence of albumin. On the other hand, treatment of HDL by phospholipase in the presence or absence of albumin produced no change in the E/A-I or A-IV/A-I ratios. Phospholipolysis of the d < 1.21 fraction resulted in a partial increment in the E/A-I ratio as compared with that observed with serum but no significant change in the A-IV/A-I ratio. Interestingly, identical treatment of human serum by pancreatic phospholipase did not result in any changes in the apoprotein distribution (Fig. 2, lanes 9 and 10).

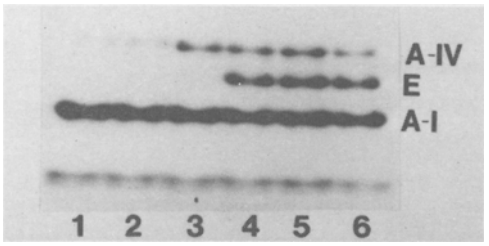

FIG. 3. SDS-PAGE of HDL isolated from rat serum treated with various concentrations of pancreatic phospholipase A_2 for 18 hr at 37 C. Final concentrations of PLASE used were: Lane 1, 0 units/ml; Lane 2, 0.005 units/ml; Lane 3, 0.05 units/ml; Lane 4, 0.5 units/ml; Lane 5, 3.0 units/ml; Lane 6, 15 units/ml.

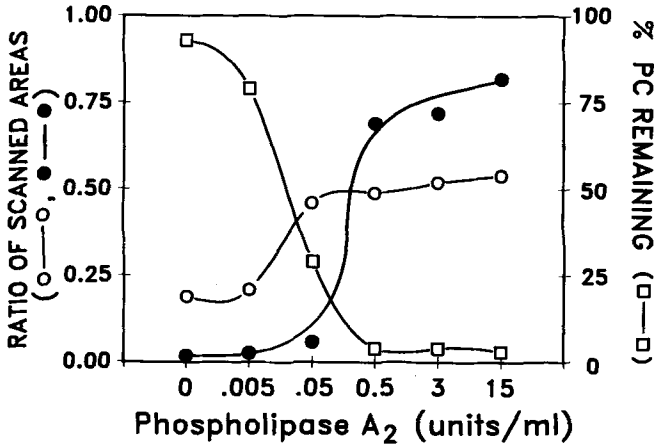

FIG. 4. Changes in apoprotein content of HDL following PLASE treatment of rat serum at various levels of enzyme activity. The SDS-PAGE gel from Figure 3 was scanned densitometrically to quantitate the changes in relative apoprotein content in these HDL. The ratio of area for the apo A-IV band to apo A-I is represented by the open circles, and the ratio for apo-E to apo A-I by the closed circles. The amount of intact phosphatidylcholine as a percentage of the sum of PC and lysoPC is represented by the open squares.

Titration of phospholipase action on apoprotein distribution. Since the standard conditions that we employed resulted in near total hydrolysis of phosphatidylcholine and phosphatidylethanolamine on the HDL, experiments were performed using a range of phospholipase activities in order to titrate the effects of phospholipase A_2 upon the HDL (Fig. 3). A quantitative treatment of these data is given in Figure 4, in which whole serum was incubated overnight with various amounts of phospholipase A_2 ranging from 0 to 15 units/ml, and the apoprotein ratios were quantitated; the extent of phospholipolysis is presented as the amount of phosphatidylcholine remaining.

DISCUSSION

Studies have shown that the HDL subpopulations are acted upon by numerous agents responsible for changes in their physicochemical properties; such agents include the LCAT enzyme, hepatic triglyceride lipase, the cholesteryl ester exchange protein and a putative "conversion factor" (1,21,22). Incubation of rat serum when LCAT is active leads to increased size of the particle (7), a shift of apolipoprotein A-IV from the lipoprotein-free portion of plasma into the HDL (8) and an increase in apo-E in the HDL fraction (7). Although the details of how these changes occur in the HDL fraction remain obscure, it appears that changes in the balance of surface and core lipid components may occur during normal metabolism of HDL in the circulation.

In these studies, pancreatic phospholipase A_2 was used as a tool to alter the surface lipids of the HDL without altering the core lipids. In executing these studies, LCAT was inhibited to prevent the formation of cholesteryl ester. Rat serum, which is devoid of the cholesteryl ester exchange protein (23), was used to avoid transfers of core lipid components during incubations and ensure that only changes in the surface lipids were being studied.

Our data demonstrate that when whole serum or a reconstituted $d > 1.063$ g/ml fraction (HDL + $d > 1.21$) is treated with phospholipase, the changes seen are similar to those observed with LCAT treatment of rat serum (7). Specifically, there is an increase in the Stokes' diameter of the particle by about 1 nm, the protein content increases as shown by chemical cross-linking and the proportions of apo-E and apo A-IV on the particle increase. These changes appear to be related to the loss of lipolysis products through binding to albumin (24), since the further addition of albumin exacerbates the observed changes and since phospholipolysis of isolated HDL or the $d < 1.21$ g/ml fraction fails to produce these changes. In fact, phospholipolysis of isolated HDL appears to cause a small decrease in Stokes' diameter, as judged by pore limit electrophoresis. Similarly, it has been reported that phospholipolysis of human HDL fractions causes negligible effects on the physicochemical properties of human HDL (9,10,25).

It appears that phospholipolysis of HDL alone causes a small loss of phospholipid components, since not all of the lysolecithin and free fatty acids produced remain on the particle (Table 2). When albumin is present, either added exogenously (Table 2) or as a serum component (Table 1), there is extensive removal of lysolecithin and free fatty acids. Treatment of HDL or $d < 1.21$ g/ml with phospholipase A_2 fails to show extensive changes in apoprotein distribution (Fig. 2), implying that this treatment causes protein uptake from the $d < 1.21$ fraction rather than a phospholipase-induced loss of apo A-I from the HDL.

It is of interest that phospholipase treatment of human serum produces no apparent changes in the apoprotein content of the human HDL as contrasted with equivalent treatment of rat serum, which results in substantial changes in the protein content of the rat HDL (Fig. 2). This appears consistent with the observation that while rat HDL undergoes marked changes in the content of apo-E and apo A-IV in response to physiological perturbations, human HDL shows a remarkably constant apoprotein composition. This suggests that the surface of human HDL differs for unknown reasons from that of rat HDL in being unsuitable for the acquisition of the A-IV or E proteins. Weinberg and Spector, for example, have shown that human apo A-IV has only a slight affinity for human HDL_3 and is displaced readily during prolonged incubations such as we have studied here (26).

Although the scanning densitometry data alone cannot distinguish increases in apo-E and apo A-IV on the

particle from loss of apo A-I, titration experiments of phospholipase action help to resolve the uncertainty (Fig. 4). If the change in apoprotein distribution was attributable to a loss of apo A-I accompanying the hydrolysis of phospholipid, we would observe a simultaneous increase in the E/A-I and the A-IV/A-I ratios. Since greater phospholipolysis is needed to alter the E/A-I ratio than to alter the A-IV/A-I ratio, we believe that this shows that E and A-IV are added separately to the particle, although some loss or redistribution of A-I also may take place.

In comparing the apoprotein distributions for HDL treated with phospholipase (Fig. 2, lanes 6 vs 5) with the $d < 1.21$ g/ml fraction similarly treated (lane 8 vs 7), it was apparent that during incubation neither apo A-IV nor apo-E were transferred to the HDL from the VLDL or LDL in any significant proportion when compared with the transfer that occurs with whole serum (lanes 2 and 3 vs 1). However, there did occur some transfer of apo-E from the $d < 1.063$ g/ml fraction, although the increment in E content of the HDL was much less than observed during phospholipolysis of whole serum (data not shown).

In summary, phospholipid treatment of whole serum and various fractions containing the HDL have demonstrated that physicochemical changes in the HDL result largely from an imbalance in the ratio of polar surface lipids and the neutral core lipids. When albumin and a source of lipid-poor apoproteins are present, there occurs a net uptake of protein and possibly some lipid to cause formation of a somewhat larger particle with a markedly different apoprotein content. Since both the LCAT and hepatic triglyceride lipase enzymes possess phospholipase activities (27,28), the changes in HDL associated with production of lysolecithin or free fatty acids and with their removal by albumin are likely to occur in vivo.

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REFERENCES

1. Eisenberg, S. (1984) *J. Lipid Res. 25,* 1017-1058.
2. Arbeeny, C.M., Edelstein, D., Freedman, S.R., and Eder, H.A. (1980) *Diabetes 29,* 774-777.
3. DeLamatre, J.G., and Roheim, P.S. (1981) *J. Lipid Res. 22,* 297-306.
4. Bar-On, H., Roheim, P.S., and Eder, H.A. (1976) *J. Clin. Invest. 57,* 714-721.
5. Nichols, A.V., Gong, E.L., and Blanche, P.J. (1981) *Biochem. Biophys. Res. Commun. 100,* 391-399.
6. Gambert, P., Lallemant, C., Athias, A., and Padieu, P. (1982) *Biochim. Biophys. Acta 713,* 1-9.
7. Swaney, J.B., Orishimo, M.W., and Girard, A. (1987) *J. Lipid Res. 28,* 982-992.
8. DeLamatre, J.G., Hoffmeier, C.A., Lacko, A.G., and Roheim, P.S. (1983) *J. Lipid Res. 24,* 1578-1585.
9. Camejo, G. (1969) *Biochim. Biophys. Acta 175,* 290-300.
10. Pattnaik, N.M., Kezdy, F.J., and Scanu, A.M. (1976) *J. Biol. Chem. 251,* 1984-1990.
11. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) *J. Clin. Invest. 34,* 1345-1353.
12. Anderson, D.W., Nichols, A.V., Forte, T.M., and Lindgren, F.T. (1977) *Biochim. Biophys. Acta 493,* 55-68.
13. Blanche, P.J., Gong, E.L., Forte, T.M., and Nichols, A.V. (1981) *Biochim. Biophys. Acta 665,* 408-419.
14. Felgenhauer, K. (1974) *Hoppe-Seyler's Z. Physiol. Chem. 355,* 1281-1290.
15. Swaney, J.B., and Kuehl, K.S. (1976) *Biochim. Biophys. Acta 446,* 561-565.
16. Swaney, J.B., and O'Brien, K. (1978) *J. Biol. Chem. 253,* 7069-7077.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
18. Markwell, M.A., Haas, S.M., Bieber, L.L., and Tolbert, N.E. (1978) *Anal. Biochem. 87,* 206-210.
19. Marinetti, G.V., Erbland, J., and Stotz, E. (1959) *Biochim. Biophys. Acta 31,* 251-252.
20. Swaney, J.B. (1980) *Biochim. Biophys. Acta 617,* 489-502.
21. Groot, P.H.E., Scheek, L.M., and Jansen, H. (1983) *Biochim. Biophys. Acta 751,* 393-400.
22. Rye, K.A., and Barter, P.J. (1984) *Biochim. Biophys. Acta 795,* 230-237.
23. Oschry, Y., and Eisenberg, S. (1982) *J. Lipid Res. 23,* 1099-1106.
24. Switzer, S., and Eder, H.A. (1965) *J. Lipid Res. 6,* 506-511.
25. Perret, B.P., Chollet, F., Durand, S., Simard, G., Chap, H., and Douste-Blazy, L. (1987) *Eur. J. Biochem. 162,* 279-286.
26. Weinberg, R.B., and Spector, M.S. (1986) *Biochem. Biophys. Res. Commun. 135,* 756-763.
27. Aron, L., Jones, S., and Fielding, C.J. (1978) *J. Biol. Chem. 253,* 7220-7226.
28. Shirai, K., Barnhart, R.L., and Jackson, R.L. (1981) *Biochem. Biophys. Res. Commun. 100,* 591-599.

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Dissolution of Cholesterol Gallstones by Bile Acids in the Prairie Dog

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The effect of chenodeoxycholic acid, ursodeoxycholic acid and hyodeoxycholic acid on gallstone dissolution was studied in the prairie dog. Cholesterol gallstones were found in all animals after feeding a semipurified diet plus 1.2% cholesterol for six wk. Gallstone regression was examined by feeding a chow diet containing the bile acids (chenodeoxycholic acid, ursodeoxycholic acid or hyodeoxycholic acid) alone (30 mg/kg/day) or in combination (chenodeoxycholic acid plus ursodeoxycholic acid) for an additional six wk. Chenodeoxycholic acid was effective in dissolving established cholesterol gallstones (two out of 16 animals still had stones) and cholesterol crystals (six out of 16 animals had crystals); the hydrophilic bile acids, ursodeoxycholic acid and hyodeoxycholic acid, were ineffective in the six-wk regression study. The lithogenic indices averaged 1.09 at the end of the induction period; all biles became unsaturated with respect to cholesterol after the six-wk regression period (group 1, 0.82; group 2, 0.66; group 3, 0.81; group 4, 0.84; group 5, 0.66). Cholesterol levels in liver, plasma and bile were elevated after the six-wk induction phase (4.59 mg/g, 610 mg/dl and 0.36 mg/ml, respectively) but returned to near normal levels after the six-wk regression period. Biliary bile acids contained increased levels of the dietary bile acid administered to each group. This experiment shows that relatively hydrophobic bile acids may be more effective than hydrophilic bile acids for gallstone dissolution during the period studied.

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The etiology, dissolution and prevention of cholesterol gallstones are topics of intense interest to clinicians. New methodologies (1) and new drugs (2,3) are under active investigation. Large scale human trials with cholelitholytic bile acids are fraught with difficulties (dose, patient population) such as those encountered in the National Cooperative Gallstone Study (4). Consequently, the use of an effective animal model to test the efficacy of new drugs before human trials remains an important area for research.

Our laboratory has used the prairie dog as an animal model to study the prevention of cholesterol gallstones (5-7). In this rodent, hydrophilic bile acids clearly were superior to hydrophobic bile acids for gallstone prevention. The hydrophilic bile acid, hyodeoxycholic acid (HDCA), inhibited gallstone formation in the presence of supersaturated bile via a phase transition mechanism (cholesterol monohydrate → liquid crystals) (6). The hydrophobic bile acid, chenodeoxycholic acid (CDCA), and the more hydrophilic acid, ursodeoxycholic acid (UDCA), caused a significant reduction in the incidence of cholesterol gallstones; a high dose of CDCA (30 mg/kg/day) and a low dose of UDCA (15 mg/kg/day) were most effective in preventing gallstones (5). The efficacy of HDCA was tested further in the prairie dog to determine whether it functioned as an effective agent for gallstone dissolution (8). In that study, using a semipurified diet only partial dissolution of cholesterol gallstones was achieved; it was hypothesized that longer time periods and perhaps larger doses of HDCA would be required for complete dissolution.

To determine whether the prairie dog could function as an effective animal model for gallstone dissolution, we tested two bile acids currently administered to man, CDCA and UDCA. These bile acids were fed in a chow diet during a six-wk regression period. In addition, we also investigated the possibility that HDCA fed in chow would be more effective as a cholelitholytic agent than in an earlier study (8) when it was fed in a semipurified diet.

MATERIALS AND METHODS

Male prairie dogs (*Cynomys ludovicianus*), bred in captivity, were purchased from R-Zoo (Neshkoro, WI). After weaning, the animals were maintained on a diet of alfalfa-corn in order to produce maximal positive response to the lithogenic diet (9). Upon arrival at our animal facility, the prairie dogs were quarantined for two wk in individual rabbit cages during which time they were fed alfalfa-corn and water ad libitum. The 59 animals then were weighed, and the diet was changed to a semipurified diet (SSD) plus 1.2% cholesterol (Teklad, Madison, WI). This diet consisted of sucrose, 56.5%; cornstarch, 13.9%; soy protein, 20.2%; corn oil, 1.2%; cellulose, 2.6%; mineral mix (Teklad 170820) and vitamin mix (Teklad 40060). Cholesterol (1.2%) was incorporated into the diet as egg yolk (0.6%) and crystalline cholesterol (0.6%). The diet was fed to all 59 animals for six wk (period 1). At wk 6, five animals (group 0) were killed to determine the incidence of cholesterol crystals and cholesterol gallstones. All five animals had biliary cholesterol crystals and gallstones (see Table 1). The remaining 54 animals were divided into five groups of similar average weights for gallstone regression, as follows: group 1, chow; group 2, chow + CDCA (30 mg/kg/day); group 3, chow + UDCA (30 mg/kg/day); group 4, chow + HDCA (30 mg/kg/day); and group 5, chow + CDCA (15 mg/kg/day) + UDCA (15 mg/kg/day). These diets were administered for six wk (period 2). The total feeding period was 12 wk. The average initial weights (wk 6) (range 833-933) and final weights (wk 12) (range 1024-1199) for the animals in groups 1-5 were not different statistically. During the 24 hr period before killing (at wk 6 or wk 12), the animals were starved to assure adequate quantities of gallbladder bile for biliary lipid analyses. All animals were anesthetized with 100 mg of ketamine hydrochloride and 20 mg of xylazine (Haver-Lockhart, Shaw-

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Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; HDCA, hyodeoxycholic acid; UDCA, ursodeoxycholic acid.

TABLE 1

Incidence of Cholesterol Gallstones, Cholesterol Crystals and Activity of Hepatic HMG-CoA Reductase[a]

Group number	Diet	No. of animals	Animals with cholesterol gallstones %	Animals with cholesterol crystals %	HMG-CoA reductase pmol/mg protein/min
0	SSD + 1.2% CH	5	100[b]	100[b]	ND
1	Chow	13	73	80	120.1 ± 19.8
2	Chow + CDCA, 30 mg/kg/day	16	12.5[c]	37.5	82.6 ± 21.9[d]
3	Chow + UDCA, 30 mg/kg/day	9	89	78	109.6 ± 42.1
4	Chow + HDCA, 30 mg/kg/day	9	78	67	196.8 ± 25.9[e]
5	Chow + CDCA, 15 mg/kg/day + UDCA, 15 mg/kg/day	7	100	100	ND

[a]CDCA, chenodeoxycholic acid; CH, cholesterol; HDCA, hyodeoxycholic acid; ND, not determined; SSD, semipurified diet; UDCA, ursodeoxycholic acid.
[b]Incidence of gallstones and crystals determined at wk 6. In all other groups, gallstone incidence was determined at wk 12.
[c]Differs significantly from groups 1 and 3-5 by chi-square, $p < 0.01$.
[d]Differs significantly from group 4, $p < 0.01$.
[e]Differs significantly from group 1, $p < 0.02$.

nee, KS), killed by exsanguination, and blood was collected for determination of serum cholesterol. The gallbladder of each animal was removed intact and inspected visually for cholesterol gallstones. Bile was aspirated with a 500 μl Hamilton syringe, and a drop was examined immediately by polarized light microscopy (Olympus MCHAP microscope equipped with camera, Olympus Corp., Lake Success, NY) to determine the presence of liquid crystals and/or cholesterol crystals. The liver was removed and weighed, aliquots were obtained for determination of liver cholesterol and preparation of microsomes (5).

Gas-liquid chromatography and gas-liquid chromatography-mass spectrometry. Gas-liquid chromatography of cholesterol in liver, plasma and bile was carried out as described (5–7). Biliary bile acids were analyzed as their methyl ester acetate derivatives using capillary gas chromatography (SPB-5 column, 15 meters, 0.25 mm ID; temperature program: 200 C - 10 min, rate of temperature increase 10 C/min to 270 C; 270 C for 20 min). Positive identification of the individual bile acids was made using a Hewlett-Packard 5992B mass-spectrometer as follows: four–ft glass column, 2 mm ID, 4 mm OD, packed with 3% SP 2250 on 100/120 mesh Supelcoport; column temperature 260 C; injector temperature 265 C; source pressure 2×10^{-6} torr; source temperature 140 C.

Reference compounds. Chenodeoxycholic acid (Canada Packers, Inc., Ontario, Canada) and ursodeoxycholic acid (Tokyo Tanabe Co., Tokyo, Japan) were analyzed as their methyl ester trimethylsilylether derivatives by gas-liquid chromatography and found to be greater than 99.5% pure. Hyodeoxycholic acid (Sigma Chemical Co., St. Louis, MO) was analyzed by the same derivatization and found to be greater than 97% pure. 3α,7α-Dihydroxy-12-keto-5β-cholanoic acid (Steroloids Inc., Wilton, NH) was used as a recovery standard for determination of biliary bile acids.

Biliary lipid composition. Gallbladder bile obtained at death immediately was centrifuged in a Beckman microfuge at 2000 X g for 10 min to remove cholesterol crystals. Aliquots of bile were used for determination of the three biliary lipids (5–7). The lithogenic indices were calculated by a computer program (10,11).

Enzyme assay. Liver microsomes were prepared, and HMG-CoA reductase activity was determined as described (5–7).

Statistical calculations. The data are reported as the average ± SEM. Analysis of variance was used on the values where the F statistic was significant, followed by Student's t-test to determine significance (12–14). Differences in incidence of gallstones and cholesterol crystals were analyzed by 2 x 2 chi-square.

RESULTS

The study reports the effects of gallstone induction with a semisynthetic lithogenic diet for six wk and gallstone regression using various bile acids fed in a chow diet for six additional wk. All five animals killed at wk six had both gallstones and crystals (Table 1); on the basis of these results and previous experiments (8), it was assumed that all of the remaining 54 animals also had stones. The lithogenic diets then were discontinued, and the remaining animals (54) were fed the regression diets (groups 2–5).

After feeding the regression diets for six wk, all animals were killed (wk 12) to determine the presence or absence of gallstones (Table 1). Animals fed chow alone showed a slow spontaneous dissolution of gallstones; 73% of these animals had stones and 80% had cholesterol crystals. Significant acceleration of stone dissolution was observed with CDCA (only 12.5% of the animals had stones and 37.5% had cholesterol crystals). The two hydrophilic bile acids tested (UDCA and HDCA) failed to produce a significant litholytic

effect (UDCA, stones 89%, crystals 78%; HDCA, stones 78%, crystals 67%). Surprisingly, when a combination of CDCA and UDCA was fed, no gallstone dissolution occurred (group 5) as had been observed with CDCA alone.

The rate–limiting enzyme of hepatic cholesterol synthesis, HMG-CoA reductase, was examined after completion of the regression period (Table 1). For animals given chow plus CDCA (group 2), the HMG-CoA reductase activity was lower by 38 units but this was not significant statistically because of the large intra-group variation. Animals fed chow plus HDCA had an enzyme activity nearly double that of the other group (196.8) (Table 1), and this activity was significantly different from those fed only chow (group 1) ($p < 0.02$).

Biliary lipid composition for the various groups is summarized in Table 2. The biles obtained from the five animals after the induction period (group 0) were supersaturated with cholesterol (L.I., 1.09). At the end of the regression period, all the biles became unsaturated with respect to cholesterol (L.I. <1.0). As expected, the mol % cholesterol was highest in group 0 (5.2 mol %). This value decreased significantly in all groups after the regression period and ranged from 2.7 to 3.2 mol %. The mol % of phospholipids and biliary bile acids in the various groups did not differ significantly ranging from 9.1 to 14.6 mol % and 80.2 to 88.2 mol %, respectively. The total lipid concentration was similar in all groups.

Cholesterol concentrations in liver, plasma and bile were determined at wk 6 and 12 (Table 3). Animals fed the gallstone induction diet had elevated cholesterol levels (wk 6) in liver, plasma and bile, averaging 4.59 mg/g, 610 mg/dl and 0.36 mg/ml, respectively. At wk 12, plasma, liver and bile cholesterol levels returned to normal and ranged from 174 to 226 mg/dl, 2.79 to 3.60 mg/g and 0.20 to 0.24 mg/ml (groups 1–5), respectively.

Biliary bile acid compositions are summarized in Table 4. The major biliary bile acids for animals fed SSD plus 1.2% cholesterol are cholic acid (CA) and CDCA. At the end of the regression period (wk 12), animals fed the chow diet alone tended to show a slight decrease in the amount of CDCA (18.3%) and a slight increase in the amount of CA (80.2%). The group of prairie dogs fed chow plus CDCA had the largest amount of CDCA in the bile (83.2%). Animals fed UDCA or HDCA showed increased concentrations of the administered bile acid (22.5% and 26.3%, respectively) but these enrichment levels were well below that for the CDCA-fed group.

DISCUSSION

The hydrophobic bile acid, chenodeoxycholic acid, fed in a chow diet was the most effective cholelitholytic agent in this study. All animals were fed a semipurified diet plus 1.2% cholesterol for six wk to induce a 100% incidence of cholesterol gallstones. This diet is known to induce gallstones in the prairie dog (15,16). The advantage of a semipurified diet is that the amount of each ingredient is known and the composition can be duplicated from batch to batch. However, we recently noted that the semipurified diet with 0.4% cholesterol produced some hepatotoxicity when fed for a period of only eight wk (moderate bile duct proliferation, portal fibrosis and inflammatory infiltration) (8). The semipurified diet plus cholesterol was more effective in producing gallstones when compared to a chow diet plus cholesterol (7). A 100% incidence of gallstones is essential for this study because the number of animals per group is relatively small. All animals are fed alfalfa-corn before the induction period to increase their susceptibility to the cholelithogenic effect of the diet. It was found that alfalfa-corn increases the activity of HMG-CoA reductase as compared to chow and increases stone incidence on lithogenic diet (9). Consequently, all animals were maintained on the alfalfa-corn diet before starting the lithogenic diet. We employed a semipurified diet for the induction period (up to wk 6) and a chow diet for the regression period (up to wk 12) to minimize liver hepatotoxicity.

TABLE 2

Effect of Bile Acids on Lipid Concentration in Prairie Dog[a]

Group number	Diet	No. of animals	Total lipid g/dl	Mol % biliary lipid: Cholesterol	Mol % biliary lipid: Phospholipid	Mol % biliary lipid: Bile acid	Lithogenic index
0	SSD + 1.2% CH	5	8.92 ± 0.66	5.2 ± 0.9	14.6 ± 1.8	80.2 ± 2.5	1.09 ± 0.16
1	Chow	13	9.06 ± 0.86	2.7 ± 0.3[b]	9.1 ± 0.9	88.2 ± 1.0	0.82 ± 0.10
2	Chow + CDCA, 30 mg/kg/day	16	10.88 ± 1.10	3.1 ± 0.3[b]	11.8 ± 1.0	85.1 ± 1.2	0.66 ± 0.05[c]
3	Chow + UDCA, 30 mg/kg/day	9	8.50 ± 1.10	3.2 ± 0.3[d]	9.5 ± 1.0	87.3 ± 1.3	0.81 ± 0.05
4	Chow + HDCA, 30 mg/kg/day	9	9.58 ± 1.11	3.2 ± 0.3[d]	9.2 ± 1.1	87.6 ± 1.8	0.84 ± 0.05
5	Chow + CDCA, 15 mg/kg/day + UDCA, 15 mg/kg/day	7	10.31 ± 1.40	3.0 ± 0.4[d]	14.3 ± 1.9	82.7 ± 2.3	0.66 ± 0.09[c]

[a]Mean ± SEM.
[b]Differs significantly from group 0, $p < 0.01$.
[c]Differs significantly from group 0, $p < 0.05$.
[d]Differs significantly from group 0, $p < 0.05$.

TABLE 3

Cholesterol Concentration in Prairie Dog at Killing[a]

Group number	Diet	No. of animals	Cholesterol		
			Liver mg/g	Plasma mg/dl	Bile mg/ml
0	SSD + 1.2% CH	5	4.59 ± 0.64[b]	610 ± 29[c]	0.36 ± 0.09
1	Chow	13	2.84 ± 0.16	225 ± 30	0.20 ± 0.03
2	Chow + CDCA, 30 mg/kg/day	16	3.60 ± 0.18	226 ± 28	0.24 ± 0.02
3	Chow + UDCA, 30 mg/kg/day	9	3.02 ± 0.13	178 ± 10	0.20 ± 0.05
4	Chow + HDCA, 30 mg/kg/day	9	3.01 ± 0.25	206 ± 20	0.22 ± 0.02
5	Chow + CDCA, 15 mg/kg/day + UDCA, 15 mg/kg/day	7	2.79 ± 0.21	174 ± 20	0.22 ± 0.04

[a]Mean ± SEM.
[b,c]Differs significantly from groups 1–5, $p < 0.01$.

TABLE 4

Biliary Bile Acid Composition at Killing (%)[a]

Group number	Diet	No. of animals	Cholic acid	Chenodeoxycholic acid	Deoxycholic acid	Lithocholic acid	6β-Hyodeoxycholic acid	Other
0	SSD + 1.2% CH	5	69.0 ± 5.8	25.2 ± 2.9	5.4 ± 1.6	0.4 ± 0.1	—	—
1	Chow	13	80.2 ± 6.2	18.3 ± 2.1	1.5 ± 1.0	0.2 ± 0.04	—	—
2	Chow + CDCA, 30 mg/kg/day	16	14.9 ± 1.5	83.2 ± 1.7	0.7 ± 0.1	1.2 ± 0.2	—	—
3	Chow + UDCA, 30 mg/kg/day	9	36.4 ± 3.2	38.3 ± 2.0	0.5 ± 0.2	2.3 ± 0.7	—	22.5 ± 2.8[b]
4	Chow + HDCA, 30 mg/kg/day	9	56.9 ± 7.8	10.1 ± 2.4	1.4 ± 0.6	—	5.3 ± 0.5	26.3 ± 3.3[c]
5	Chow + CDCA, 15 mg/kg/day + UDCA, 15 mg/kg/day	7	12.6 ± 2.9	67.3 ± 2.6	0.5 ± 0.3	2.5 ± 0.6	—	17.1 ± 1.5[b]

[a]Mean ± SEM.
[b]Ursodeoxycholic acid.
[c]Hyodeoxycholic acid.

In accord with previous observations in our laboratory, the activity of hepatic microsomal HMG-CoA reductase was greater in animals fed laboratory chow than those fed a (cholesterol-free) semipurified diet (7). The addition of CDCA to the chow regression diet produced a 35% decrease in enzyme activity (this trend was not statistically significant), while UDCA produced a negligible change (Table 1). The failure to show a significant lowering of HMG-CoA reductase levels after CDCA administration probably is ascribable to large intra-group variations that also have been observed by others (17). Because the prairie dogs currently in use are either trapped in the wild or are the direct offspring of such animals, they are likely to be quite heterogeneous, genetically and metabolically. The very hydrophilic HDCA stimulated the activity of the rate-limiting enzyme almost two-fold, presumably because this bile acid interferes with the intestinal absorption of cholesterol (7). However, in these experiments with animals maintained on low-cholesterol chow the reduced absorption of cholesterol was not reflected in a lowering of serum or liver cholesterol levels. This is in contrast with the striking reduction in tissue cholesterol levels produced by HDCA in previous experiments with cholesterol-fed animals (6).

Tissue cholesterol levels analyzed at wk 6 showed a significant response to the increased dietary cholesterol. Liver, plasma and bile cholesterol concentrations were elevated in all animals. These levels returned to near normal values in all groups after the six-wk regression period. Thus, the administration of the chow diet (with and without bile acids) reverses the elevations in cholesterol in the tissues studied.

The biles of all animals in groups 1–5 had become unsaturated with respect to cholesterol (L.I. <1.0) (wk 12) but only CDCA was able to dissolve the cholesterol gallstones. The relative ineffectiveness of the hydrophilic bile acids UDCA and HDCA fed either alone or in combination (CDCA plus UDCA) may be ascribed to a number of factors. First, a relatively short regression period of six wk was chosen, which may have been inadequate to demonstrate appreciable dissolution. Second, although all biles were unsaturated at the end of 12 wk it is conceivable that the rates of desaturation were not identical and were slower for the more hydrophilic bile acids. Third, we

were unable to demonstrate the formation of liquid crystalline phases in the biles of the prairie dogs treated with UDCA or HDCA, suggesting that the phase transition from crystalline cholesterol monohydrate to cholesterol/phospholipid mesophase was inoperative or at least nonobservable under the conditions employed. Fourth, after the administration of UDCA this bile acid accounted for only 23% of total biliary bile acids. The failure of administered UDCA to accumulate in the gallbladder bile of prairie dogs had been observed in gallstone prevention experiments (5) and was attributed to the partial conversion of UDCA to CDCA. In addition, HDCA, which was not transformed into CDCA, accounted for only about 26% of total biliary bile acids; epimerization of HDCA to the hydrophilic 3α,6β-dihydroxy-5β-cholanoic acid also was very small (5%) as compared to previous studies (20-30% [6]). Therefore, it seems possible that with the chow diet used for the regression studies, hydrophilic bile acids may not be absorbed as efficiently as they were when given in cholesterol-containing semipurified diets.

Our studies suggest some important differences that should be considered when examining bile acids for gallstone prevention vs gallstone dissolution. First, in prevention studies the bile acid is administered simultaneously with the start of the lithogenic diet. Since no gallstones are present at time 0, the drug can either prevent bile saturation (L.I. <1.0) or solubilize large amounts of cholesterol in supersaturated bile (in vesicles, L.I. >1.0). Chenodeoxycholic acid and hyodeoxycholic acid operate via these respective mechanisms. For gallstone dissolution, other factors may be operative. These include the number and size of the gallstones present at the start of the regression period. Thus, bile acids that can desaturate bile most rapidly, such as hydrophobic compounds, should be most effective in the short term. In the present study, only CDCA dissolved gallstones in six wk even though the biles of all groups had become unsaturated with respect to cholesterol (L.I. <1.0). Igimi and Carey reported from their in vitro studies that chenodeoxycholic acid should dissolve gallstones faster than ursodeoxycholic acid (18). We now know that factors other than micelle formation play a role in gallstone dissolution (18). Thus, it is well known that in man UDCA and UDCA/CDCA combination dissolve stones at least as rapidly as CDCA. These drugs presumably all act by lowering the cholesterol saturation of bile (19,20). Desaturation of bile no doubt favors stone dissolution but no longer can be considered the sole, necessary condition for achieving dissolution. The formation of liquid crystalline phases of high cholesterol content characteristic of hydrophilic bile acids is a second mechanism potentially favoring cholelitholysis. A multiplicity of factors, in addition to type and dosage of bile acid, and duration of treatment may play a role in gallstone dissolution in the prairie dog model. Not only bile composition but also structure and surface characteristics of the stones remain important considerations in development of a satisfactory prairie dog model for gallstone dissolution.

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REFERENCES

1. Sauerbruch, T., Delius, M., Paumgartner, G., Holl, J., Wess, O., Weber, W., Hepp, W., Ing, Dr., and Brendel, W. (1986) *N. Engl. J. Med., 314*, 818–822.
2. Loria, P., Carulli, N., Medici, G., Menozzi, D., Salvioli, G., Bertolotti, M., and Montanari, M. (1986) *Gastroenterology 90*, 865–874.
3. Lanzini, A., Pigozzi, G., Facchinetti, D., Castellano, M., Bettini, I., Beschi, M., and Muiesan, G. (1986) *Curr. Therapeut. Res. 39*, 8–18.
4. Schoenfield, L.J., Lachin, J.M., The Steering Committee, and The National Cooperative Gallstone Study Group (1981) *Ann. Intern. Med. 95*, 257–282.
5. Cohen, B.I., Singhal, A.K., Stenger, R.J., May-Donath, P., Finver-Sadowsky, J., McSherry, C.K., and Mosbach, E.H. (1984) *Hepatology 4*, 300–307.
6. McSherry, C.K., Mosbach, E.H., Cohen, B.I., Une, M., Stenger, R.J., and Singhal, A.K. (1985) *Am. J. Surg. 149*, 126–132.
7. Cohen, B.I., Mosbach, E.H., McSherry, C.K., Stenger, R.J., Kuroki, S., and Rzigalinski, B. (1986) *Hepatology 6*, 874–880.
8. Cohen, B.I., Mosbach, E.H., McSherry, C.K., Rzigalinski, B., and Kuroki, S. (1986) *Lipids 21*, 575–579.
9. Cohen, B.I., Mosbach, E.H. and McSherry, C.K. (1986) *Hepatology 6*, 1134.
10. Carey, M.C. (1978) *J. Lipid Res. 19*, 945–955.
11. Kuroki, S., Cohen, B.I., Carey, M.C., and Mosbach, E.H. (1986) *J. Lipid Res. 27*, 442–444.
12. Roscoe, J.T. (1975) in *Fundamental Research for the Behavioral Sciences*, 2nd ed., pp. 254–262, 292–303, 309–322, Holt, Reinhart and Winston, New York, NY.
13. Dunnett, C.W. (1964) *J. Am. Stat. Assn. 50*, 1096–1121.
14. Snedecor, G.W. (1946) in *Statistical Methods*, 4th ed., pp. 56–81, 182–184, Iowa State University Press, Ames, IA.
15. Chang, S.-H., Ho, K.-J., and Taylor, C.B. (1973) *Arch. Pathol. 93*, 417–426.
16. Brenneman, D.E., Connor, W.E., Forker, E.L., and DenBesten, L. (1972) *J. Clin. Invest. 51*, 1495–1503.
17. Howell, J.H., Marsh, M.E., Thiel, E.S., and Holzbach, R.T. (1983) *Biochim. Biophys. Acta 753*, 32–39.
18. Igimi, H., and Carey, M.C. (1981) *J. Lipid Res. 22*, 254–270.
19. Leuschner, U. (1986) *Med. Klin. 81*, 217–222.
20. Tint, G.S., Salen, G., and Shefer, S. (1986) *Gastroenterology 91*, 1007–1018.

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Normalization of High Density Lipoprotein in Fish Eye Disease Plasma by Purified Normal Human Lecithin: Cholesterol Acyltransferase

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Plasma from a patient with fish eye disease has been enriched with autologous high density lipoproteins (HDL) and supplemented with highly purified normal human plasma lecithin:cholesterol acyltransferase (LCAT). Incubation of such plasma at 37 C in vitro resulted in normalization of its low HDL cholesteryl ester percentage, from 23% to 79%, associated with a two-fold increase in both the cholesteryl ester and triglyceride contents of the HDL fraction, as compared to incubation experiments with absent or heat-inactivated purified normal LCAT.

The normalization of the HDL cholesteryl ester percentage induced by incubation with purified normal LCAT also was accompanied by an increase in the size of the original fish eye disease HDL particles, which had a mean mass of 115 kd, to HDL particle populations with mean particle masses ranging from 130-220 kd, depending on the concentration of purified LCAT in the incubate. Both HDL cholesterol esterification and particle enlargement were abolished completely by the LCAT inhibitor DTNB and by heat inactivation of the purified normal LCAT. The results give further evidence that fish eye disease is an α-LCAT deficiency.
Lipids 23, 225–229 (1988).

Fish eye disease is a rare hereditary hypoalphalipoproteinemia showing plasma high density lipoprosein (HDL) cholesterol and apolipoprotein A-I concentrations that both are reduced by about 90% as compared to what is normal (1,2). Only about 20% of the total cholesterol of the HDL particles is esterified in this disease (3). By contrast, normal HDL contain about 80% of its total cholesterol in the esterified form. A further abnormality of the fish eye disease HDL relates to its apparent mean particle size or mass, which is 115 kilodalton (kd) as compared to mass values ranging from 130 to 350 kd for normal HDL particles (2). Recently, we demonstrated that fish eye disease plasma has a specific lack of lecithin:cholesterol acyltransferase (LCAT) activity that is present in normal plasma and specifically seems to catalyze the esterification of the free cholesterol of HDL (4). Although cholesterol of HDL in fish eye disease plasma is not esterified by autologous LCAT, the free cholesterol of the combined plasma very low density (VLDL) and low density lipoproteins (LDL) is esterified at an apparently normal rate in this disease as revealed by both esterification rate experiments and compositional analyses of VLDL and LDL cholesteryl ester percentages and fatty acids (4).

To explain the unique plasma cholesterol esterification situation in fish eye disease, we introduced the hypothesis that there exist two LCAT activities of different substrate specificities in normal plasma (4,5). The enzyme activities specific for the free cholesterol of HDL or that of combined VLDL and LDL were denoted α- and β-LCAT, respectively. Thus, fish eye disease was classified as an α-LCAT deficiency syndrome. In a series of papers, we showed that neither impaired or deficient plasma lipid transfer proteins (6–8) nor qualitatively defective apolipoproteins (9) seem likely causes of the apparent specific lack of HDL cholesterol esterification in fish eye disease plasma.

In this study, we have supplemented fish eye disease plasma with normal human LCAT purified more than 20,000–fold over its occurrence in normal plasma and with retained α- and β-LCAT activities (10). The effect of this enzyme on the particle size and relative cholesteryl ester content of the fish eye disease HDL is reported herein.

EXPERIMENTAL PROCEDURES

General methods. The only living Swedish fish eye disease patient No. 1, now 73 years old, has been characterized in detail (2). Venous blood was taken from the patient in the mornings after an overnight fast into vacutainer plastic tubes containing disodium ethylenediamine tetraacetate (EDTA). The tubes immediately were placed into crushed ice. Plasma was recovered within 30 min by low speed centrifugation at 2 C and stored in crushed ice. Merthiolate®, normally added to plasmas at our work with lipoproteins, was omitted. Free cholesterol and cholesteryl esters were quantified enzymatically in duplicate directly on aliquots of plasma or on the isolated lipoprotein fractions obtained after ultracentrifugation using the kits 14106, 14107 and 14108 (E. Merck, Darmstadt, FRG). Triglyceride concentrations were estimated in duplicate on aliquots of isolated lipoproteins as previously described (2). The coefficients of variation (number of estimates in parentheses) were 3.5% (57), 3.1% (57) and 2.7% (75) for between-run reproducibility of determinations of free cholesterol, esterified cholesterol and triglycerides, respectively. Polyacrylamide gradient gel electrophoresis was performed as reported (2). Ultracentrifugations were carried out in a Beckman Model L5-55 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) using a 50.3 Ti rotor and 6 ml Bell-Top and 2 ml open Ultra-Clear® tubes at 1 C. After completed ultracentrifugation, tube fractions were collected by the tube-slicing technique, using a

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Abbreviations: DTNB, disodium 5,5-dithiobis-(2-nitrobenzoate); EDTA, ethylenediamine tetraacetic acid; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein(s); VLDL, very low density lipoprotein(s).

Beckman Tube-Slicer in combination with a special template to yield fractions of desired volumes. Concentrated fish eye disease HDL were prepared as described and dialyzed at 5 C against 0.15 mol/l sodium chloride solution (8). Briefly, the density of fish eye disease plasma was raised to 1.063 kg/l by the addition of solid sodium bromide. After centrifugation, each tube (6 ml) was sliced to give a bottom fraction of 3.37 ml. The density of this fraction was raised to 1.21 kg/l. New centrifuge tubes (6 ml) were filled with this solution and run for 67 hr at 50,000 rpm (7.2×10^8 g x min). Concentrated HDL was obtained from the 1 ml top of the tube (supernatant d=1.21 kg/l). For analysis of the lipid composition of the HDL fraction of native fish eye disease plasma, these lipoproteins were isolated as a 1.063–1.21 kg/l density fraction by means of sequential ultracentrifugations as reported earlier (12). Double immunodiffusion against anti-human apolipoprotein D, which we obtained as a gift from P. Alaupovic, Oklahoma City, was performed in Ouchterlony 1% agarose gel plates.

Purified normal human LCAT was obtained by covalent chromatography as described in detail (10). The enzyme accepted the free cholesterol of both heat-inactivated HDL-deficient or combined VLDL- and LDL-deficient plasmas as substrate when a modified Glomset and Wright method (4,5) was used for enzyme activity tests. The highly purified enzyme was electrophoretically and immunologically free of apolipoproteins A-I, A-II, C and E, and the enzyme did not show zones that could be assigned to known lipid transfer proteins (11) on SDS-gelelectrophoresis. Supplementation with apolipoprotein A-I was necessary to transform lecithin:cholesterol liposomes into substrates for the purified LCAT. The final enzyme purification on hydroxyapatite (10) was performed batch-wise just before the intended use of the enzyme that had a specific activity of more than 43,000 units/mg corresponding to a purification degree of more than 20,000 over its occurrence in plasma (10).

Incubation experiments. Native fish eye disease plasma (1 ml) was mixed with concentrated autologous fish eye disease HDL (1 ml) and either purified LCAT in 1 mmol/l phosphate buffer, pH 6.8, containing 4 mmol/l 2-mercaptoethanol but with no other stabilizer (0.2 ml) or with the same amount of a solution of this enzyme after 60 min heat inactivation at 56 C or with 0.15 mol/l sodium chloride (0.2) ml containing 4 mmol/l 2-mercaptoethanol as control. Incubations performed in the presence of DTNB (disodium 5,5-dithiobis-2(-nitrobenzoate)) as LCAT inhibitor were made by addition of phosphate buffered 100 mmol/l DTNB, pH 7.4 to the relevant incubation mixture to give a 2 mmol/l final concentration. The final volumes (2.24 ml) of all incubation mixtures were equalized by addition of 0.15 mol/l sodium chloride. Incubations were performed at 0 and 37 C in the dark under nitrogen atmosphere for 24 hr.

The concentrations of free cholesterol and cholesteryl esters of native fish eye disease plasma, of the fish eye disease plasma HDL fraction and of the concentrated fish eye disease HDL (see general methods) used in the incubation experiments with purified normal LCAT are compiled in Table 1.

TABLE 1

Concentrations of Free, Esterified and Total Cholesterol (mmol/l of Incubation Mixture) and Cholesteryl Ester Percentage of Native Fish Eye Disease Plasma, its HDL Fraction and of Isolated Concentrated Fish Eye Disease HDL, Prepared for Incubation Experiments

	FC	CE	TC	CE
Specimen from fish eye disease		(mmol/l)		(%)
Concentrated HDL	0.33	0.10	0.43	23
Native plasma	2.76	3.73	6.49	57
HDL in native plasma	0.10	0.03	0.13	23

FC, free cholesterol; CE, cholesteryl esters; TC, total cholesterol as sum of free and esterified cholesterol.

After completed incubation, each incubation mixture was parted into two equal volumes. From one of the volumes, the total lipoproteins were isolated by ultracentrifugation at density 1.21 kg/l, obtained by addition of sodium bromide solution of d=1.46 kg/l, for 65 hr and 145,000 x g. The other volume was utilized for isolation of the HDL fraction (d=1.063-1.21 kg/l) of the incubation mixture by means of sequential density ultracentrifugation as reported earlier (12).

Isolated lipoprotein fractions were submitted to polyacrylamide gradient gel electrophoresis and cholesterol analyses as described above.

RESULTS

The concentration of free, esterified and total cholesterol and the cholesteryl ester percentage of the isolated total plasma lipoproteins ($d < 1.21$ kg/l) obtained after 24 hr of incubation at 37 C of fish eye disease plasma or such plasma mixed with purified normal LCAT are compiled in Table 2. This table also shows corresponding concentration values obtained at total LCAT inhibition by DTNB and at experiments with added heat-inactivated purified enzyme.

When fish eye disease plasma was incubated without addition of normal LCAT, the free cholesterol content of the total plasma lipoproteins decreased by 0.27 mmol per l of incubation mixture as compared to the same incubation mixture stored at 0 C. Concomitantly, the cholesteryl ester content of the total lipoproteins in relation to total cholesterol increased from 57% to 65% in incubations with active autologous β-LCAT esterifying the free cholesterol of combined VLDL and LDL in the mixture.

Incubation of fish eye disease plasma with purified normal LCAT at a subnormal amount of 0.8 mg per l (normal plasma concentration is 4-8 mg/l) of incubation mixture or with twice this amount of enzyme resulted in increases in the cholesteryl ester percentages of the total plasma lipoproteins to 70 and 73%, respectively. This latter increase was reflected by a decrease of the free cholesterol of about 0.4 mmol per l of incubation mixture. When the LCAT inhibitor DTNB was included in the incubation mixture of fish

TABLE 2

Concentrations of Free, Esterified and Total Cholesterol (mmol/l of Incubation Mixture) and Cholesteryl Ester Percentage of Total Lipoproteins (d < 1.21 kg/l) Isolated after Incubation for 24 hr at 37 C of Fish Eye Disease Plasma Enriched with Autologous HDL and Purified Normal LCAT at Different Conditions

Additions to mixture of fish eye disease plasma and HDL	FC	CE	TC	CE
	(mmol/l)			(%)
0.15 M NaCl at 0 C	1.24	1.65	2.89	57
0.15 M NaCl	0.97	1.78	2.75	65
Normal LCAT (1 μg)	0.85	1.96	2.81	70
Normal LCAT (1 μg) + DTNB[a]	1.16	1.67	2.83	59
Heat-inact. normal LCAT (1 μg)	0.96	1.79	2.75	65
Normal LCAT (2 μg)	0.76	2.03	2.79	73

FC, free cholesterol; CE, cholesteryl esters; TC, total cholesterol as sum of free and esterified cholesterol.
[a]2 mmol/l of incubation mixture.

TABLE 3

Concentrations of Free, Esterified and Total Cholesterol and Triglycerides (mmol/l of Incubation Mixture) and Cholesteryl Ester Percentage of Isolated HDL (d = 1.063 - 1.21 kg/l) Isolated after Incubation for 24 hr at 37 C of Fish Eye Disease Plasma Enriched with Autologous HDL and Purified Normal LCAT at Different Conditions

Additions to mixture of fish eye disease plasma and HDL	FC	CE	TC	TG	CE
	(mmol/l)				(%)
0.15 M NaCl at 0 C	0.17	0.05	0.22	0.057	23
0.15 M NaCl	0.04	0.04	0.08	0.043	50
Normal LCAT (1 μg)	0.05	0.09	0.14	0.070	64
Normal LCAT (1 μg) + DTNB[a]	0.03	0.03	0.06	0.022	50
Heat-inact. normal LCAT (1 μg)	0.04	0.04	0.08	0.050	50
Normal LCAT (2 μg)	0.03	0.11	0.14	0.073	79

FC, free cholesterol; CE, cholesteryl esters; TC, total cholesterol as sum of free and esterified cholesterol; TG, triglycerides.
[a]2 mmol/l of incubation mixture.

eye disease plasma and purified LCAT, the values for the contents of free cholesterol and cholesteryl esters and for the cholesteryl ester percentage were close to corresponding values obtained for fish eye disease plasma stored at 0 C (Table 2).

Addition of heat-inactivated purified normal LCAT to fish eye disease plasma, followed by incubation at 37 C yielded essentially the same values for free cholesterol concentration and cholesteryl ester percentage as were obtained for fish eye disease plasma without addition of purified normal enzyme.

After incubation of the different mixtures, their HDL fractions were also isolated and analyzed for free, esterified and total cholesterol and triglycerides. These results are presented in Table 3. Compared to fish eye disease plasma stored at 0 C for 24 hr, the free and total cholesterol contents of HDL from all mixtures incubated at 37 C were reduced. The reduction of total cholesterol of HDL was 64% (from 0.22 to 0.08 mmol/l) in incubation experiments with both fish eye disease plasma alone and with such plasma mixed with purified normal LCAT and incubated in the presence of 2 mmol/l DTNB for total LCAT inhibition. The corresponding reduction of total HDL cholesterol in incubation experiments with fish eye disease plasma mixed with heat-inactivated purified normal LCAT was 72% (from 0.22 to 0.06 mmol/l). In all these latter three experiments, the cholesteryl ester percentage of the isolated HDL was 50% after completed incubation, as calculated from total cholesterol contents that were of the same order of magnitude in all these three incubation experiments.

When purified normal LCAT was added to fish eye disease plasma, the HDL cholesteryl ester percentage increased to 64 and 79% on incubation when 0.8 and 1.6 mg of enzyme per l of incubation mixture, respectively, was used. The reduction of total cholesterol of HDL was 36% (from 0.22 to 0.14 mmol/l) in these experiments, as calculated from the total HDL cholesterol content obtained at incubation at 0 C. In addition, there was a net increase in cholesteryl ester content of the isolated HDL after incubation, from about 0.05 to 0.10 mmol/l when added purified normal LCAT was active (Table 3).

Incubation experiments with fish eye disease plasma with and without LCAT inhibitor or with such plasma that had been mixed with heat-inactivated purified normal LCAT demonstrated HDL particles of molecular masses around 115 kd (Fig. 1). When fish eye disease plasma was incubated with purified normal LCAT at a subnormal concentration of 0.8 mg per l of incubate, HDL particles of apparent masses around 130 kd were produced, and all particles of apparent masses of 115 kd disappeared (Fig. 1). At an enzyme concentration of 1.6 mg per l of incubation mixture, still larger HDL particles, around 200 kd appeared.

DISCUSSION

Fish eye disease plasma lacks the property to esterify the free cholesterol of HDL (4). It recently was demonstrated that when such native plasma was incubated at 37 C, both free cholesterol and phospholipids disappeared from its HDL as a manifestation of the lack of esterification of the cholesterol of this lipoprotein (7). No increase in HDL cholesteryl ester content occurred in these experiments with native fish eye disease plasma (7). As the originally present cholesteryl ester content remained unchanged in the HDL fraction after incubation, its percentage of esterified cholesterol increased from 20% to 50%. However, this increase was not accompanied by an increase in the apparent mean particle size of HDL (7) in contrast to what is observed when there is a net increase in the cholesteryl ester content and percentage of both normal and fish eye disease HDL induced by incubation with α-LCAT present in normal, lipoprotein depleted plasma (4,13,14). Instead, it could be demonstrated by

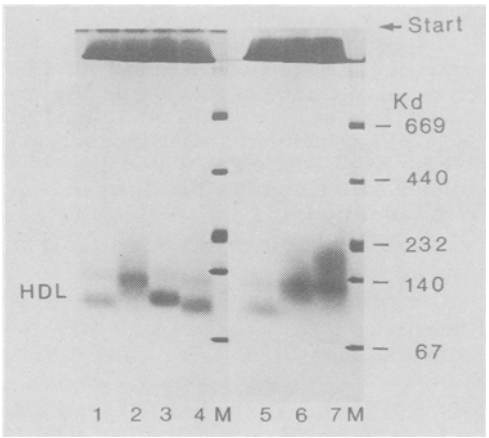

FIG. 1. Electrophoretogram of polyacrylamide gradient gel electrophoresis of total lipoproteins (d < 1.21 kg/l) isolated from different mixtures containing native fish eye disease plasma enriched with autologous HDL and incubated for 24 hr at 37 C with the following additives per l of incubation mixture (same incubation mixtures as described in Tables 2 and 3).
1 and 5, 0.15 mol/l sodium chloride; 2 and 6, 0.8 mg of purified normal LCAT; 3, 0.8 mg of purified normal LCAT and 2 mmol of DTNB; 4, 0.8 mg heat-inactivated purified normal LCAT; 7, 1.6 mg of purified normal LCAT; M, Molecular mass markers (Pharmacia Biotechnology International); Kd, Kilodalton.
Zones are stained by Amido Black.

polyacrylamide gradient gel electrophoresis that the HDL of native fish eye disease plasma gradually disappeared after prolonged incubation at 37 C, probably as a result of reduction of particle size as discussed in detail earlier (7). It seems reasonable to conclude that on incubation the free cholesterol and phospholipids of fish eye disease HDL diffuse to the plasma VLDL and LDL and are utilized at these lipoproteins as substrates for an active β-LCAT.

In this study, in agreement with previous observations (7) incubation at 37 C for 24 hr resulted in a disappearance of the free cholesterol of the HDL fraction of fish eye disease plasma enriched with autologous HDL. Supplementation with HDL was made to get enough material for all different analyses. Consonant with previous observations (7) the disappearance of free cholesterol from HDL also occurred in incubation experiments with LCAT inhibitor (Table 3).

In incubation experiments with fish eye disease plasma without LCAT inhibitor, there was a net increase in cholesteryl ester content of the total lipoproteins (d<1.21 kg/l), as compared to the LCAT inhibited situation, due to esterification of the free cholesterol of combined VLDL and LDL, demonstrating a functioning β-LCAT activity in accordance with previous findings (4,5). The decrease in free cholesterol of the fish eye disease HDL particles resulted in an increase in the cholesteryl ester percentage from 23% to 50% but did not increase the apparent HDL particle size or mass.

We recently developed a new procedure for the purification of normal human LCAT (10) with retained α- and β-LCAT activities as previously defined (4,5). This enzyme, which was purified more than 20,000-fold over its occurence in plasma by covalent chromatography on Thiopropyl-Sepharose accepted artificial lecithin-cholesterol (molar ratio 4:1) liposomes as substrates only after supplementation with purified apolipoprotein A-I.

Incubation of this highly purified enzyme with fish eye disease plasma at 37 C for 24 hr resulted in an increase in the cholesteryl ester percentage of the total lipoproteins from 65% to more than 70%. This net increase in cholesteryl esters of the total lipoproteins was reflected by a net increase in esterified cholesterol in HDL isolated after incubation. The cholesteryl ester content increased two-fold as compared with that of fish eye disease HDL from plasma not supplemented with normal purified LCAT. The induced esterification of the free cholesterol of the fish eye disease HDL also strongly counterbalanced the HDL particle degradation process with disappearance of more than 60% of its total cholesterol content. Furthermore, a net increase in the triglyceride content of HDL isolated after incubation at 37 C compared with what was found at incubation at 0 C also could be demonstrated. This increase in HDL triglyceride content is expected to occur due to a functioning bidirectional cholesteryl ester-triglyceride transfer between HDL and lipoproteins of lower densities and induced by the increase in the cholesteryl ester content of the HDL on incubation with normal purified LCAT with α-LCAT activity. All the incubation-induced effects of purified normal LCAT on fish eye disease HDL and total lipoprotein cholesterol were abolished completely by heat inactivation of the purified LCAT before it was added to the incubation mixtures (Tables 2 and 3).

In addition to apolipoprotein A-I, apolipoprotein D, which also resides in normal HDL, has been reported to be involved as a cofactor in the LCAT-mediated esterification process of the free cholesterol of plasma (15). This apolipoprotein, whose role in the esterification process is disputed (16), also seems to be the most persistently remaining impurity in even the highest purified LCAT preparations (17).

In fish eye disease plasma, both apolipoprotein A-I, which is normal qualitatively (9), and apolipoprotein D are present, although in strongly reduced amounts (2). As demonstrated in Figure 2, fish eye disease HDL produced a precipitation arc on double immunodiffusion against anti-apolipoprotein D. The presence of apolipoprotein D in fish eye disease HDL, combined with the observed inability of heat-inactivated purified normal LCAT to effect HDL cholesterol esterification make it reasonable to conclude that the demonstrated incubation-induced normalization of the fish eye disease HDL cholesteryl ester content is caused by an α-LCAT activity characteristic of the normal purified LCAT used in this study and not by traces of apolipoprotein D, which is temperature resistant, in the purified LCAT preparation.

The normalization of the cholesteryl ester content of isolated total lipoproteins and HDL, observed after incubation of fish eye disease plasma supplemented with purified normal LCAT, was found to be accompa-

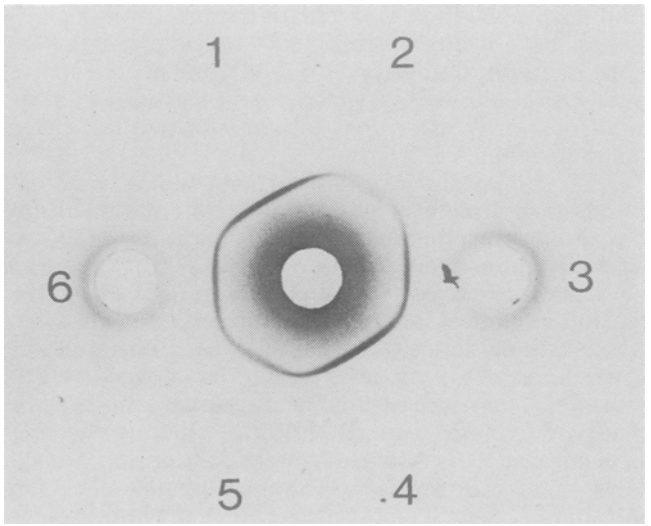

FIG. 2. Double immunodiffusion in Ouchterlony 1% agarose gel plates. Inner well-contained anti-apolipoprotein D.
Outer wells contained: 1 (5 μl) and 4 (10 μl), normal HDL; 2 (5 μl) and 5 (10 μl), fish eye disease HDL; 3 (5 μl) and 6 (10 μl), normal plasma.
Precipitation lines are stained with Coomassie Brilliant Blue R.

nied by a normalization of the apparent mean particle size or mass of these HDL. Incubations with the subnormal LCAT concentration yielded a rather homogeneous population of particles that were larger than those of native fish eye disease HDL (Fig. 1). Mean particle masses of the HDL increased as a function of increasing purified LCAT concentration in the incubation mixture. The particle enlargement induced by the action of normal purified LCAT, resulted in HDL particles of the same size or mass as found for small HDL_3 particles in normal plasma (Fig. 1). It seems that when the cholesteryl ester percentage of HDL reaches a value of about 60%, accompanied by a decrease in free cholesterol content of the HDL particle surface, whose decrease might render the particle less rigid (18), an HDL particle fusion process may start (8). A fusion process in transformation of small model discoidal lipoprotein complexes to HDL_3-like species previously has been demonstrated by Nichols et al. (19). The effect of the purified normal LCAT on fish eye disease HDL was abolished completely by the LCAT inhibitor DTNB or by heat inactivation of the purified normal enzyme.

These findings demonstrate that α-LCAT activity of normal purified LCAT is necessary and sufficient for the initiation of this HDL particle enlargement in fish eye disease plasma, although additional factors affecting HDL particle size might be involved at later steps of the process in this as well as in normal plasma (14,20).

The results of this study clearly show that fish eye disease plasma, consistent with earlier findings in experiments with lipoprotein-depleted plasma fractions (4,5), lacks an enzyme activity (α-LCAT) that is present in normal highly purified LCAT, although the free cholesterol of the combined VLDL and LDL is esterified at an apparently normal rate in this condition. Supplementation of fish eye disease plasma in vitro with purified normal LCAT followed by incubation at 37 C for 24 hr resulted in normalization of both the cholesteryl ester content and the apparent mean particle size or mass of the fish eye disease HDL.

ACKNOWLEDGMENT

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REFERENCES

1. Carlson, L.A., and Philipson, B. (1979) *Lancet II*, 921–923.
2. Carlson, L.A. (1982) *Eur. J. Clin. Invest. 12*, 41–53.
3. Carlson, L.A., and Holmquist, L. (1983) *Acta Med. Scand. 213*, 177–182.
4. Carlson, L.A., and Holmquist, L. (1985) *Acta Med. Scand. 218*, 189–196.
5. Carlson, L.A., and Holmquist, L. (1985) *Acta Med. Scand. 218*, 197–205.
6. Calvert, G.D., and Carlson, L.A. (1983) *Acta Med. Scand. 213*, 253–254.
7. Holmquist, L., and Carlson, L.A. (1987) *Lipids 22*, 305–311.
8. Holmquist, L., and Carlson, L.A. (1987) *Acta Med. Scand. 221*, 283–289.
9. Marshall, T., Williams, K.M., Holmquist, L., Carlson, L.A., and Vesterberg, O. (1985) *Clin. Chem. 31*, 2032–2035.
10. Holmquist, L. (1987) *J. Biochem. Biophys. Meth. 14*, 323-333.
11. Albers, J.J., Tollefson, J.H., Chen, C.H., and Steinmetz, A. (1984) *Arteriosclerosis 4*, 49–58.
12. Carlson, K. (1973) *J. Clin. Pathol. 5* (Suppl), 32–37.
13. Nichols, A.V., Gong, E.L., and Blanche, J. (1981) *Biochim. Biophys. Res. Commun. 100*, 391–399.
14. Carlson, L.A., and Holmquist, L. (1983) *Atherosclerosis 47*, 305–306.
15. Fielding, P.E., and Fielding, C.J. (1980) *Proc. Natl. Acad. Sci. USA 77*, 3327–3330.
16. Albers, J.J., Cheung, M.C., Ewens, S.L., and Tollefson, J.H. (1981) *Atherosclerosis 39*, 395–409.
17. Marcel, Y.L., Vézina, C.A., Weech, P.K., Tercé, F., and Milne, R.W. (1985) in *Lipoprotein deficiency Syndromes*, (Angel, A., and Frohlich, J., eds.) pp. 163–179, Plenum Press, New York, NY.
18. Ellerbe, P., Murphy, R.B., and Rose, H.G. (1985) *Chem. Phys. Lipids 38*, 275–285.
19. Nichols, A.V., Blanche, P.J., Gong, E.L., Shore, V.G., and Forte, T.M. (1985) *Biochim. Biophys. Acta 834*, 285–300.
20. Gambert, P., Lallemant, C., Athias, A., and Padieu, P. (1982) *Biochim. Biophys. Acta 713*, 1–9.

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Cholesterol Synthesis in the Pathogenesis of Lithocholic Acid-induced Cholestasis

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Lithocholic acid (LCA)-induced intrahepatic cholestasis is associated with increased de novo synthesis of hepatic cholesterol and augmented cholesterol content of the liver cell plasma membrane fraction enriched in bile canalicular complexes (BCM). To determine whether inhibition of cholesterol synthesis could prevent LCA-induced cholestasis, adult male Wistar rats were treated daily with the hypocholesterolemic agents, clofibrate (250 mg/kg) or mevinolin (25 mg/kg), for one, two or four days. After bile duct cannulation and bile collection for one hr, the animals were injected intravenously with 120 μmoles/kg of LCA or its carrier (albumin). Cholesterol synthesis was measured in liver homogenates, and its contribution to the BCM was estimated. LCA reduced bile flow by 51%, 35% and 25% after clofibrate pretreatment for one, two and four days, respectively, and by 51%, 30% and 42% in mevinolin-pretreated animals after one, two and four days. In control animals, cholesterol synthesis and the contribution of newly synthesized cholesterol in the BCM were increased after LCA injection. However, despite that cholesterol synthesis and the contribution of newly synthesized cholesterol in the BCM were reduced in drug-pretreated rats, LCA injection caused a relative increase in these parameters of a magnitude similar to that observed in controls. Thus, the ability of LCA injection to augment de novo cholesterol synthesis and its transport to the BCM may be an important pathogenetic step in the development of cholestasis.
Lipids 23, 230-233 (1988).

Cholestasis generally may be defined as a primary disturbance in bile formation. While current understanding of the pathogenesis of intrahepatic cholestasis remains fairly rudimentary, considerable attention has been focused on monohydroxy bile acid-induced cholestasis (1-4). Monohydroxy bile acids have been identified in the meconium and urine of newborns (5-8), and de novo synthesis of these bile acids in fetus and newborn infant has been suggested (9-10). More recently, the concentration of lithocholic acid (LCA), a monohydroxy bile acid, was found to be increased in the bile of adult patients treated with chenodeoxycholic acid for gallstones (11-13), and there have been reports of elevated biliary LCA levels in subjects receiving total parenteral nutrition (14-15). It is of interest that the hepatic structural alterations noted in some of these patients are somewhat similar to those produced in animals by LCA (13). Therefore, elucidation of the pathogenesis of LCA-induced cholestasis could prove useful in the management of prevention of cholestasis in these high-risk patients.

Existing evidence indicates that LCA enhances de novo cholesterol synthesis in microsomes as well as cholesterol transport from the microsomes to the bile canalicular membrane (BCM) into which it becomes incorporated (16). Cholesterol accumulation in the BCM then could increase the membrane's viscosity and thereby decrease its permeability to water, resulting in cholestasis (16-20). Therefore, it has been suggested that LCA-induced cholestasis could be prevented by agents that interfere with hepatic cholesterol synthesis and consequently reduce cholesterol content of the BCM. This hypothesis was tested with clofibrate (ethyl-p-chlorphenoxy isobutyrate), a hypolipidemic drug that suppresses the conversion of acetate into cholesterol in liver homogenates (21), and mevinolin (1,2,6,7,8 α - hexahydro-β,δ-dihydroxy-2,6-dimethyl-8-(2-methyl-1-oxobutoxy-1-naphtalene hepatonic acid δ-lactone, MK-803), a highly potent specific competitive inhibitor of 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG-CoA), the rate-limiting enzyme in cholesterol biosynthesis (22).

MATERIALS AND METHODS

Effect of clofibrate and mevinolin on lithocholate-induced cholestasis. Male Wistar rats (High Oak Ranch, Montreal, Québec) with an average body weight of 200 g were kept under controlled conditions of humidity and lighting with free access to water and Purina lab chow. They were divided into three groups. The two treatment groups were given per os for one, two or four days either clofibrate (gift from Ayerst Laboratories, Montreal, Canada) at a daily dose of 250 mg/kg body weight or mevinolin (gift from Merck Sharp & Dohme, Montreal, Canada) at a daily dose of 25 mg/kg body weight. The third group served as untreated controls. Within one hr after the last oral dose of clofibrate or mevinolin, a biliary fistula was created under nembutal anesthesia, and bile was collected for one hr (0-60 min collection). Half of the animals of each of the three groups were injected intravenously with either 120 μmol LCA-Na/kg body weight (Calbiochem-Boehring Corp., La Jolla, CA); purity 90% as checked by thin layer chromatography (TLC) and gas liquid chromatography (GLC) or with the LCA carrier, 7.5% albumin in saline. The LCA injected was labeled with ^{14}C-LCA (Amersham, Oakville, Ontario); specific activity 55 μCi/mmole, 98% of the ^{14}C radioactivity was recovered with LCA when analyzed with thin layer chromatography (TLC) using a solvent system made up of iso-octane/ethyl-acetate/acetic acid/N-butanol (10:5:1.5:1.5). Bile was col-

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Abbreviations: BCM, bile canalicular complexes; GLC, gas liquid chromatography; LCA, lithocholic acid; TLC, thin layer chromatography.

lected for one hr (60–120 min collection) following the injection of LCA or albumin solution, and its volume was measured gravimetrically. The rats remained under anesthesia during bile collections (120 min), and body temperature was maintained at 36.5 C with a heat lamp thermostatically controlled. At the end of the experiment, the livers were homogenized in bicarbonate buffer, and specimens were solubilized in tissue solubilizer and counted for radioactivity. Blood samples were taken from rats injected with ^{14}C-LCA, and their radioactivity was determined. The methodology for the radioactivity measurements in liver tissues and blood has been reported earlier by this laboratory (16). Experimental and control animals were studied simultaneously to exclude diurnal variation in bile acid and cholesterol metabolism.

Effect of clofibrate and mevinolin on cholesterol synthesis and liver cell plasma membrane composition. These experiments were designed to determine the effects of the hypocholesterolemic drugs on hepatic cholesterol synthesis and on cholesterol content of liver cell plasma membrane fractions, utilizing the recently described ^{14}C/^{3}H ratio technique (16,20). Because results from the first phase of the study showed that maximum protection against LCA-induced cholestasis was obtained after four days of clofibrate and two days of mevinolin treatment, two groups of rats received these agents for such periods of time before half of them were injected with 120 μmol LCA/kg body weight or its carrier (albumin 7.5% in saline). A third group of rats untreated with hypocholesterolemic drugs served as controls, half the animals were injected with LCA and the other half with the LCA carrier as previously described. Sixteen hours before the LCA or albumin injection, all the animals were injected intraperitoneally with 25 μCi of ^{3}H cholesterol/100 g body weight (1,2-^{3}H cholesterol, New England Nuclear, Montreal; specific activity 50 mCi/mmol) to label the hepatic cholesterol pool. The rats were later injected IV with 10 μCi of ^{14}C mevalonic acid/100 g body weight (New England Nuclear, Montreal; specific activity 49 mCi/mmol) at the same time of the injection of LCA or its carrier to label the newly synthesized cholesterol. An hour later, the animals were killed. Their livers were washed with an infusion of 20 ml of 0.1 mM $NaHCO_3$ and 0.05 mM $CaCl_2$ buffer and were homogenized in the same buffer (1:2, W/V). A pellet of sodium hydroxide was added to each ml of homogenate, and the mixture was hydrolyzed for one hr at 70 C. Neutral lipids then were extracted with petroleum ether and the extracts dried under nitrogen at 60 C. The lipids were solubilized with 1 ml of methanol, and total cholesterol was measured by GLC (23), after which 1 ml of 5% digitonin (Fisher Scientific Co., Fairlawn, NJ) was added to precipitate the cholesterol. The mixture then was centrifuged at 3000 g at 4 C; the pellet was dissolved in 2 ml of hot methanol; and 0.5 ml was taken for the measurement of radioactivity, using a Beckman liquid scintillation system (Model L5 700) equipped with an external standard. The specific activities of cholesterol were determined, and the ^{14}C/^{3}H ratio was calculated. This ratio represents the relative contribution of newly synthesized cholesterol to the total cholesterol pool from the time of injection of LCA or its carrier up to the end of the experiment (one hr), because ^{3}H specific activity of hepatic cholesterol was in a steady state between 12–24 hr after ^{3}H cholesterol injection.

The rest of the liver homogenate was used for the processing of liver cell plasma membrane fractions, as described (2,16). Two fractions were obtained, one enriched in bile canalicular structures, the other mainly containing sheets and vesicles derived from the sinusoidal surface of the cell. Lipids were extracted with chloroform/methanol (2:1), total cholesterol was determined by gas liquid chromatography (GLC), and the ^{14}C and ^{3}H radioactivities were estimated as described for liver homogenate extract. The ^{14}C/^{3}H ratios of the specific activity of cholesterol in the membrane fractions then were calculated. The data were evaluated statistically by Student's t-test and a P-value of <0.05 was considered statistically significant.

RESULTS

Body and liver weight. The percentage of liver to body weight ratio was increased significantly ($p < 0.05$) after two and four days of clofibrate treatment (% liver to body weight ratios were 4.29 ± 0.28, 4.70 ± 0.18 and 4.88 ± 0.45 for one, two and four days of treatment, respectively). Mevinolin administration had no such effect.

Bile flow. In clofibrate-treated rats, basal bile flow (0–60 min collection period) decreased after one and two days of treatment as compared with controls but this trend disappeared after four days (Table 1). A similar response pattern was observed following treatment with mevinolin.

LCA in control animals that had not been treated with clofibrate or mevinolin decreased bile flow by 58% within one hr following its injection (Table 2). Similarly, in clofibrate-treated rats for one, two and

TABLE 1

Bile Flow in Rats given Clofibrate and Mevinolin alone or with LCA

Treatment	Bile flow (μl/min/g/liver) 0–60 min before LCA	Bile flow (μl/min/g/liver) 60–120 min after LCA
Control + albumin	1.75 ± 0.20	1.81 ± 0.17
Control + LCA	1.94 ± 0.24	0.82 ± 0.23[a,b]
Clofibrate 1 day + LCA	1.48 ± 0.17	0.72 ± 0.17[a,b]
Mevinolin 1 day + LCA	1.46 ± 0.15[a]	0.71 ± 0.46[a,b]
Clofibrate 2 days + LCA	1.44 ± 0.17[a]	0.93 ± 0.26[a,b]
Mevinolin 2 days + LCA	1.74 ± 0.21	1.21 ± 0.28[a,b]
Clofibrate 4 days + LCA	1.79 ± 0.36	1.36 ± 0.51
Mevinolin 4 days + LCA	1.61 ± 0.40	0.92 ± 0.51[a,b]

[a]Significantly different ($p < 0.05$) from the controls injected with albumin only.
[b]Significantly different ($p < 0.05$) from the 0–60 min values.
Values represent the means ± SD of eight animals except for control + albumin and control + LCA where the number of animals was four.

TABLE 2

Percentage of Distribution of LCA Radioactivity in Various Tissues

Treatment	Serum	Liver	Bile
Control + LCA	8.5 ± 4.9	48.5 ± 3.5	43.0 ± 8.5
Clofibrate 1 day + LCA	2.5 ± 1.9[a]	44.5 ± 13.5	53.0 ± 14.4
Clofibrate 2 days + LCA	2.9 ± 1.9[a]	31.1 ± 8.4[a]	67.0 ± 10.0[a]
Clofibrate 4 days + LCA	2.8 ± 1.9[a]	26.3 ± 14.3[a]	70.9 ± 11.2[a]
Mevinolin 1 day + LCA	5.0 ± 1.0	44.0 ± 6.9	51.0 ± 7.3
Mevinolin 2 days + LCA	3.5 ± 2.6	29.6 ± 13.6[a]	66.9 ± 17.8[a]
Mevinolin 4 days + LCA	2.3 ± 1.1[a]	29.4 ± 10.3	68.3 ± 11.7[a]

Values are the means ± SD of eight animals except for control + LCA, the number of animals was four.
[a]Significantly different ($p < 0.05$) from the control group.

four days, bile flow was reduced by 51, 35 and 25%, respectively. At four days, bile flow was significantly greater ($p < 0.05$) than in controls injected with LCA.

In rats given mevinolin, LCA reduced bile flow by 51, 30 and 43% after one, two and four days of treatment, respectively. Although bile flow after two days of mevinolin administration was significantly higher ($p < 0.05$) than in control animals receiving LCA, this difference was not seen after four days of treatment (Table 1.)

Distribution of LCA in liver, serum and bile. Table 2 shows the percentage distribution of radioactive LCA in liver, serum and bile. In control animals, LCA was distributed equally in liver and bile (48 and 43%, respectively), whereas the serum contained only 8% of the dose injected. Both clofibrate and mevinolin increased the recovery of LCA in bile, and this was accompanied by proportionate reductions in liver and serum.

Cholesterol content and relative cholesterol synthesis. As seen in Table 3, none of the treatments significantly influenced the total cholesterol content of liver homogenates or the $^{14}C/^{3}H$ ratio.

Cholesterol content in the BCM was increased by 30% after LCA injection in control animals (no drug pretreatment). Clofibrate or mevinolin treatments for four or two days, respectively, reduced cholesterol content in the BCM by 30% (compared to control animals not treated with LCA). Injection of LCA in the drug-pretreated animals did not result in an increase in the membrane cholesterol (compared to control animals injected with LCA). The cholesterol content of the PM fraction after LCA injection was not affected in control animals (not treated with drugs); cholesterol was reduced, however, by 30–50% in drug treated animals irrespective of LCA or albumin injection.

The $^{14}C/^{3}H$ ratio, which indicates the relative contribution of the newly synthesized cholesterol in the total cholesterol content of the membrane, was increased in the BCM by 30% after the injection of LCA in controls (not treated with drugs). This ratio was reduced by a similar percentage in clofibrate and mevinolin treated rats. Injection of LCA in the later groups increased the $^{14}C/^{3}H$ ratio to the level seen in control animals (not injected with LCA).

In the PM fraction, the $^{14}C/^{3}H$ ratio was increased after LCA injection in control animals. This ratio was reduced in the drug treated groups whether injected with LCA or albumin. The difference in the $^{14}C/^{3}H$ ratios between BCM and PM may indicate a different rate of contribution of newly synthesized cholesterol to cholesterol content in these two membrane fractions.

DISCUSSION

The purpose of these studies was to test the hypothesis that the increased de novo synthesis of cholesterol after LCA treatment and its accumulation in the canalicular membrane are important pathogenetic steps in the development of LCA-induced cholestasis.

It was assumed that inhibition of cholesterol synthesis by the hypocholesterolemic drugs, clofibrate and mevinolin, may prevent cholesterol accumulation in the canalicular membrane and the cholestatic response to LCA. The data obtained in these studies do not support this hypothesis; despite the fact that clofibrate and mevinolin both reduced the relative cholesterol synthesis and cholesterol content in the BCM (Table 3), bile flow was not improved significantly after LCA injection (Table 1) (with the exception of clofibrate treatment for four days in which bile flow was reduced by 25% vs 58% in control. But, clofibrate effect was not time- or dose-dependent (unpublished data). However, in clofibrate and mevinolin-treated rats, LCA caused an increase in the relative contribution of de novo cholesterol synthesis in the BCM (C^{14}/H^{3} 0.08 in clofibrate vs 0.11 in clofibrate + LCA injection and 0.08 in mevinolin vs 0.10 in mevinolin + LCA injection). This increase was similar to that obtained in control rats (C^{14}/H^{3} 0.12 in controls vs 0.15 in LCA injection). These data suggest that the ability of LCA injection to increase de novo synthesis of cholesterol and its transport to the BCM may be an important step in the pathogenesis of LCA-induced cholestasis.

Recently, it was shown in vitro that cholestatic bile acids such as LCA and its sulfate and glucuronide derivatives possess higher calcium-binding properties than noncholestatic bile acids (24,25). This finding raised the possibility that a disturbance of cellu-

TABLE 3

Cholesterol Content and Relative Cholesterol Synthesis

Treatment	Homogenate		BCM		PM	
	Cholesterol (μmol/g liver)	$^{14}C/^{3}H$	Cholesterol (nmol/mg protein)	$^{14}C/^{3}H$	Cholesterol (nmol/mg protein)	$^{14}C/^{3}H$
Control + albumin	21.2 ± 1.8	0.10 ± 0.098	460 ± 29	0.12 ± 0.011	334 ± 28	0.07 ± 0.009
Control + LCA	21.5 ± 2.4	0.12 ± 0.097	602 ± 57[a]	0.15 ± 0.013[a]	334 ± 23	0.12 ± 0.01[a]
Clofibrate 4 days + albumin	20.9 ± 1.7	0.06 ± 0.008	314 ± 18[a,b]	0.08 ± 0.009[a,b]	244 ± 27[a,b]	0.04 ± 0.006[a,b]
Clofibrate 4 days + LCA	21.1 ± 2.4	0.09 ± 0.010	311 ± 23[a,b]	0.11 ± 0.020[b]	162 ± 19[a,b]	0.05 ± 0.00[a,b]
Mevinolin 2 days + albumin	21.4 ± 2.1	0.05 ± 0.007	324 ± 21[a,b]	0.08 ± 0.012[a,b]	258 ± 21[a,b]	0.04 ± 0.004[a,b]
Mevinolin 2 days + LCA	21.5 ± 1.8	0.08 ± 0.009	318 ± 19[a,b]	0.10 ± 0.011[b]	189 ± 18[a,b]	0.05 ± 0.00[a,b]

Values are the means ± SD of four samples per group.
BCM refers to membrane fractions enriched in bile canalicular complexes (bile canaliculi and lateral membranes).
PM refers to membrane fractions containing vesicles derived mainly from sinusoidal and lateral membranes of hepatocytes.
The $^{14}C/^{3}H$ ratio is used as a measure of the newly synthetized cholesterol (ref. 16).
[a]Significantly different ($p < 0.05$) from control injected with albumin.
[b]Significantly different ($p < 0.05$) from control injected with LCA.

lar calcium homeostasis could occur after exposure to LCA with pathologic consequences. If this is proven in vivo, it remains to be determined to what extent changes in cellular calcium could modulate the mobilization of cholesterol from microsomes to canalicular membranes that occurs after LCA injection.

The increased biliary secretion of ^{14}C-LCA in drug-pretreated rats could be due to increased metabolites of LCA. Preliminary experiments show that 92% of ^{14}C LCA was secreted unchanged in controls, while only 50% was secreted in clofibrate-treated rats. However, if this was the case it does not seem that the increased secretion of LCA (or its metabolites) was significant enough to prevent its cholestatic effect.

In summary, this and other studies (2,16,20) from our laboratory confirm the association between the cholesterol synthesis and the cholestatic effect of LCA. Furthermore, it shows that under the present experimental conditions hypolipidemic drugs do not prove useful in prevention of LCA-induced cholestasis.

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REFERENCES

1. Javitt, N.B. (1975) in *Jaundice* (Goresky, C.A., and Fisher, M.M., eds.), pp. 401–409, Plenum Press, New York, NY.
2. Kakis, G., and Yousef, I.M. (1978) *Gastroenterology 75,* 595–607.
3. Fisher, M.M. (1979) in *Toxic Injury of the Liver* (Farber, E., and Fisher, M.M., eds.), pp. 155–192, Marcell Dekker, New York, NY.
4. Kakis, G., and Yousef, I.M. (1980) *Gastroenterology 78,* 1402–1411.
5. Sharp, H., Carey, J.B., Peller, J., and Krivit, W. (1968) *Pediatr. Res. 2,* 293.
6. Sharp, H., Peller, J., Carey, J.B., and Krivit, W. (1971) *Pediatr. Res. 5,* 274–279.
7. Anderson, K.E., Kok, E., and Javitt, N.B. (1972) *J. Clin. Invest. 51,* 112–117.
8. Back, P. (1973) *Clin. Chim. Acta 44,* 199–207.
9. Lester, R. (1983) *J. Pediatr. Gastroenterol. Nutr. 2,* 335–336.
10. Lester, R., Pyrek, J., Little, J.M., and Adcock, E.W. (1983) *J. Pediatr. Gastroenterol. Nutr. 2,* 335–364.
11. Danzinger, R.G., and Hofmann, A.F. (1972) *N. Engl. J. Med. 1,* 286.
12. Allan, R.N., Thistle, J.L., and Hofmann, A.F. (1976) *Gut 17,* 413–419.
13. Phillips, M.J., Fisher, R.L., Anderson, D.W., Lan, S.P., Lachin, J.M., Boyer, J.L., and the Steering Committee for the National Cooperative Gallstone Study Group (1983) *Hepatology 3,* 209–220.
14. Farell, M.K., Balistreri, N.F., and Suchy, J.F. (1982) *J. Parenteral and Enteral Nutr. 6,* 30–33.
15. Fouin-Fortunet, H., LeQuernec, L., Erlinger, S., Lerebours, E., and Colin, R. (1982) *Gastroenterology 82,* 932–937,
16. Yousef, I.M., and Tuchweber, B. (1984) *Biochim. Biophys. Acta 796,* 336–344.
17. Papahadjopoulos, D. (1976) in *Lipids* (Paoletti, R., Porcelati, G., and Jacini, G., eds.), pp. 187–196, Raven Press, New York, NY.
18. Cooper, R.A. (1978) *J. Supramol. Struct. 8,* 413–430,
19. Shattil, S.J., and Cooper, R.A. (1978) *Prog. Hemost. Thromb. 4,* 59–68,
20. Yousef, I.M., Lewittes, M., Tuchweber, B., Roy, C.C., and Weber, A.M. (1984) *Biochim. Biophys. Acta 796,* 345–353.
21. Cayen, N.M., and Dvornik, D. (1970) *Can. J. Biochem 48,* 1022–1023.
22. Tobert, J.A., Hitzenberger, G., Kutovetz, W.R., Holmes, I.B., and Jones, K.H. (1982) *Atherosclerosis 41,* 61–65.
23. Yousef, I.M., Fisher, M.M., Myher, J.J., and Kiksis, A. (1976) *Anal. Biochem 75,* 538–544.
24. Oelberg, D.G., Dubinsky, N.P., Adcock, E.W., and Lester, R. (1984) *Am. J. Physiol. 247,* G112–G115.
25. Oelberg, D.G., Mohan, D.C., Little, J.N., Adcock, E.W., and Lester, R. (1984) *J. Clin. Invest. 73,* 1507–1514.

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Butter-enriched Diets Reduce Arterial Prostacyclin Production in Rats

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Rats were fed diets containing 10%, 30% or 50% energy as fat derived predominantly from butter or lard. The protein content of the diets was maintained at 20%. After three weeks on the diets, the rats were killed and the following parameters measured: prostacyclin production in vitro from abdominal aorta and mesenteric artery; platelet aggregation to ADP and thrombin; fatty acid composition of the phospholipids in plasma, thoracic aorta and liver; smooth muscle reactivity and release of endothelial derived relaxing factor (EDRF) from aortic endothelium stimulated by acetylcholine. There was no significant effect of increasing fat content of the diets (neither lard nor butter) on platelet aggregation. In contrast, prostacyclin production in both the mesenteric artery and the abdominal aorta fell in a concentration-dependent manner in the butter-supplemented rats. However, no effect on prostacyclin production was detected in arteries from the lard-supplemented animals. The effects of the diets on prostacyclin (PGI_2) production correlated very well with the changes in plasma, aortic and liver phospholipid arachidonic acid (AA) and eicosapentaenoic acid (EPA) contents. AA decreased in a concentration-dependent manner in the rats fed the butter-enriched diets but did not change in those fed the lard-enriched diets, whereas EPA rose in a concentration-dependent manner in the butter-fed rats and was unchanged in the lard-fed animals. The clear-cut effects of the butter-enriched diets on aortic phospholipid fatty acid composition and aortic PGI_2 production were accompanied by a significant reduction in smooth muscle relaxation to EDRF. These results indicate that in the rat, enrichment of the diet with butter can reduce the concentration of AA and increase that of EPA in plasma and tissue phospholipids with a parallel reduction in arterial PGI_2 production and EDRF.

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It now is accepted widely that high levels of saturated fat in the diet increase the risk of coronary heart disease (1,2). The effects of dietary saturated fat appear to be mediated through two major pathways: elevated plasma cholesterol levels, resulting in increased risk of atherosclerosis (1), and increased thrombosis tendency (3). The mechanism by which thrombosis tendency is increased is not well understood. Renaud and coworkers have reported that dietary saturated fat is associated with an increase in the concentration of the fatty acid 20:3n-9 in plasma and platelet lipids in rats and humans (4,5), a phenomenon that usually occurs only in essential fatty acid deficiency (6). They attributed the increased thrombin-induced platelet aggregation associated with dietary saturated fat to the presence of this fatty acid (5). However, it also is possible that the increased levels of 20:3n-9 are a marker of other changes in plasma and tissue fatty acid composition that may affect directly the production of the arachidonic acid (AA)-derived prostanoids (prostacyclin [PGI_2] and thromboxane A_2) and thereby modulate thrombosis tendency. To examine this possibility, the aim of this study was to determine the effects of increasing the proportion of two different dietary fats (butter and lard) on vascular PGI_2 production and platelet aggregation, and plasma, aortic and liver phospholipid fatty acid composition in the rat. In addition, the effects of increased dietary fat (butter) on aortic smooth muscle reactivity and the release of endothelial derived relaxing factor (EDRF) were assessed.

METHODS

Animals. Male Sprague-Dawley rats weighing 120–170 g were used in all studies. They were housed in a temperature-controlled room with a 12-hr light-dark cycle. Food and water were provided ad libitum and food consumption and body weights recorded three times each week.

Diets. The nutrient composition of the experimental diets is shown in Table 1 and their fatty acid composition is given in Table 2. The diets were prepared from whole meal flour and skim milk powder (providing all the carbohydrate and protein) and lard or butter (providing most of the fat). The proportion of energy derived from protein was constant in all diets, while the proportions of fat and carbohydrate varied inversely. The P/S ratio fell as the proportion of fat rose on both the lard and butter diets, however the fall was more pronounced on the butter diet as the P/S ratio of butter is much lower than that of lard. Vitamins and minerals were added to reach the levels recommended by the American Institute of Nutrition (7).

Platelet aggregation. After three weeks on the diet, the rats were anesthetized with sodium pentobarbitone (50 mg/kg intraperitoneally), and blood was taken by cardiac puncture into 3.8% sodium citrate (9 vol blood: 1 vol citrate). The citrated blood was centrifuged

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Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; EC_{10}, EC_{50}, EC_{90}, the concentrations that will give 10%, 50% or 90% response (dilation or constriction), respectively; EDRF, endothelial-derived relaxing factor; EPA, eicosapentaenoic acid; KRB, Krebs Ringer bicarbonate buffer; NO, nitric oxide; PGI_2, prostacyclin I_2; PPP, platelet-poor plasma; PRP, platelet-rich plasma; P/S ratio, the ratio of polyunsaturated to saturated fatty acids; PUFA, polyunsaturated fatty acid; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; 20:3n-9, a 20 carbon fatty acid with three double bonds, the first one being nine carbons (n-9) from the methyl end.

TABLE 1

Composition of the Experimental Diets

	Butter			Lard		
Constituents (g/100 g)	10%	30%	50%	10%	30%	50%
Whole meal flour	73.1	42.5	4.6	73.5	43.8	4.8
Skim milk powder	20.0	38.7	61.3	20.1	39.8	64.9
Butter	2.5	14.4	29.7	—	—	—
Lard	—	—	—	2.0	12.0	25.9
Choline bitartrate	0.1	0.1	0.1	0.1	0.1	0.1
Mineral mix	3.3	3.3	3.3	3.3	3.3	3.3
Vitamin mix	1.0	1.0	1.0	1.0	1.0	1.0
Composition (% energy)						
Protein	20	20	20	20	20	20
Carbohydrate	70	50	30	70	50	30
Fat	10	30	50	10	30	50
P/S ratio	0.56	0.10	0.05	0.54	0.24	0.19

TABLE 2

Fatty Acid Composition of the Diets

	Butter			Lard		
Fatty acid	10%	30%	50%	10%	30%	50%
8:0	1.2	1.4	1.9	—	—	—
10:0	2.1	2.77	4.2	—	—	—
12:0	2.4	2.8	3.4	—	—	—
14:0	6.5	10.5	11.3	1.8	1.8	1.7
14:1	0.5	0.7	0.8	—	—	—
15:0	0.8	1.2	1.2	—	—	—
16:0	24.7	30.7	29.8	25.1	30.9	27.0
16:1	1.4	1.6	2.9	1.2	0.8	2.1
17:0	0.8	1.0	0.6	0.9	0.6	0.6
18:0	9.4	14.9	14.0	14.4	16.5	17.9
18:1	23.0	25.1	25.9	32.5	36.3	40.6
18:2n-6	24.6	5.7	2.4	21.3	11.1	8.2
18:3n-3	2.2	1.1	1.2	1.6	1.0	0.9
20:0	0.2	0.3	0.2	0.3	0.3	0.3
20:1	0.2	0.3	0.2	0.9	0.7	0.7
Saturates	48.1	65.5	66.6	42.5	50.1	47.5
Monoenes	25.1	27.7	29.8	34.6	37.8	43.4
Polyenes	26.8	6.8	3.6	22.9	12.1	9.1
n-6/n-3 ratio	11.2	5.2	2.0	13.3	11.1	9.1

at 150 × g for 10 min at room temperature to prepare platelet-rich plasma (PRP). After removal of the PRP, the remaining blood was centrifuged for 15 min at 3000 × g at room temperature to obtain platelet-poor plasma (PPP). The platelet count of the PRP was determined using a Coulter Counter (Model SII) and adjusted with PPP to give a count of 300,000 platelets/μl. Platelet aggregation measurements were performed with a Payton dual-channel aggregometer: 450 μl PRP was stirred at 1000 rpm at 37 C and 50 μl of the appropriate aggregating agent added: ADP (2 and 5 μM), thrombin (1 and 2 U/ml) or AA (6.4 mM). Platelet aggregation was monitored continuously for four min. To quantitate platelet aggregation, the maximum change in light transmittance through PRP was expressed as a percentage of the light transmittance through PPP. Five min after the addition of the aggregating agent, a 200 μl aliquot was mixed with 200 μl of ethanol, centrifuged and the supernatant stored at -20 C for subsequent radioimmunoassay of thromboxane B_2 (TXB_2), the stable metabolite of thromboxane A_2 (TXA_2) (8).

Vascular PGI_2 production. Immediately after blood collection, the rat was perfused with Krebs Ringer Bicarbonate buffer (KRB), pH 7.4, containing 5.5 mM glucose, to flush out the remaining blood, leaving the artery preparations free of adhering blood clots. The mesenteric artery and abdominal aorta were removed carefully, cleared of connective tissue and cut into rings. These rings were incubated in 4 ml of KRB containing 5.5 mM glucose at 37 C for three hr in a shaking water bath and 100 μl aliquots taken at zero time and 15, 30, 60, 120 and 180 min later. These samples were stored at -20 C and the time course of PGI_2 production determined subsequently by radioimmunoassay of its stable metabolite, 6-keto $PGF_{1\alpha}$ (9). At the end of the incubation, the rings were removed, blotted on filter paper and weighed to obtain the "wet weight." In order to control for the presence of any cross-reacting material interfering with the RIA of 6-keto-$PGF_{1\alpha}$ produced during the incubation of arteries, we incubated aortic rings from butter-fed rats (10% and 50%) in the presence and absence of indomethacin (10 μg/ml). Indomethacin completely prevented the production of any immuno-reactive material from these aortea, confirming the specificity of the assay for 6-keto-$PGF_{1\alpha}$.

Aortic smooth muscle reactivity and the release of EDRF. Rats were stunned and the thoracic aorta carefully dissected free of surrounding tissue. The aorta was pinned down at either end on a silastic rubber-coated dish filled with cold, oxygenated Krebs' solution composition (in mM) Na^+ 144, K^+ 5.9, Ca^{++} 2.5, Mg^{++} 1.2, Cl^- 128.7, HCO_3^- 25, SO_4^{2-} 1.2, glucose 11, EDTA 0.27 aerated with a gas mixture of 95% O_2 and 5% CO_2. Two three-ring segments each 3 mm long were cut transversally from the aorta. Each ring was suspended on two surgical stainless steel Z-shaped support wires 500 μm diameter in a heated organ bath. One wire was attached to an isometric force transducer (Grass FTO3C), the second wire to an acrylic leg mounted on a micrometer screw gauge. After 30 min

equilibration, the micrometer was advanced to stretch the artery in steps every minute. The passive force-diameter relationship was determined from the measurements as described (10). The internal diameter (D) and circumference (L) of the vessel was calculated for an equivalent transmural pressure of 100 mm Hg. The artery was released slightly to a circumference of 0.9 L and left at this passive stretch for the remainder of the experiment. These initial steps were taken to ensure that each artery segment was under similar resting passive tone before drug addition. From each rat, one ring was contracted to a steady level of active force by phenylephrine hydrochloride 1 μM, then relaxed by the cumulative addition of acetylcholine bromide at 0.5 log unit increments (from 10–1000 nM) to test the EDRF activity. Relaxation responses were averaged at fixed concentrations of each.

Fatty acid composition of plasma aortic, and liver phospholipids. Lipid extracts were prepared from plasma, thoracic aorta and liver by chloroform/methanol extraction (11). Phospholipids were separated by thin layer chromatography and the concentration of the component fatty acids determined by capillary gas liquid chromatography using heptadecanoic acid as an internal standard as described (12). The identification of 20:3n-9 was based on identical retention times with 20:3n-9 isolated from essential fatty acid deficient rat liver when separated on a 50 m x 0.32 mm bonded phase capillary column coated with CP Sil 88. On this column, 20:3n-9 was separated clearly from 20:2n-6, 20:3n-6 and 22:0.

Statistical analyses. Differences between groups were established using analysis of covariance or Student's unpaired t-test. Results are expressed as mean ± SEM.

MATERIALS

Pentobarbitone sodium was purchased from Civa Chemicals (Hornsby, N.S.W., Australia), ADP, TXB_2 standard, 6-keto $PGF_{1\alpha}$ standard, acetylcholine bromide and indomethacin were from Sigma Chemical Co. (St. Louis, MO); thrombin (Fibrindex, human) from Orthodiagnostic Systems Inc. (Raritan, NJ); arachidonic acid and heptadecanoic acid were from NuChek Prep (Elysian, MN), TXB_2 antibody was a gift from Laurence Levine, Brandeis University (Waltham, MA); 6-keto-$PGF_{1\alpha}$ antibody was a gift from L.C. Best, University of Sheffield (Sheffield, U.K.); tritiated 6-keto-$PGF_{1\alpha}$ and TXB_2 were from Amersham International (Amersham, U.K.); bonded phase capillary column from Chrompak (Middelburg, The Netherlands); and phenylephrine hydrochloride from Stirling Pharmaceuticals (Sydney, Australia).

RESULTS

Whether the rats were fed butter or lard diets did not affect their weight gain. At 10%, 30% and 50% butter diets, the rats gained 7.3 ± 0.1, 7.0 ± 0.3 and 6.3 ± 0.3 g/day, while with the 10%, 30% and 50% lard diets they gained 7.5 ± 0.1, 7.1 ± 0.2 and 6.1 ± 0.3 g/rat/day. However, in both dietary studies (butter and lard), there was a reduction in weight gain as the fat content of the diet increased. This probably was due in large part to the major changes in salt and water balance that occur on low carbohydrate diets (13,14).

There was no effect of either type of dietary fat on ADP-induced platelet aggregation (Table 3). Although there was a trend towards increased platelet aggregation in response to the higher concentration of thombin (2 U/ml) with increasing fat intake (both lard and butter), it was not statistically significant. Thromboxane production by platelets in vitro was not influenced by either type of dietary fat (Table 3).

The effect of increasing the level of butter in the diet on the time-course of PGI_2 production by the abdominal aorta is shown in Figure 1. PGI_2 production over the three hr was reduced consistently as the proportion of dietary fat rose from 10% to 50% of energy (analysis of covariance, $p < 0.001$). Subsequent results are presented as the PGI_2 production in the first hour

TABLE 3

The Effects of the Diets on Platelet Aggregation and Thromboxane A_2 Production in Platelets (See Methods)

	Butter			Lard		
Platelet aggregation (%)	10% n = 10	30% n = 10	50% n = 10	10% n = 5	30% n = 4	50% n = 3
ADP 2 μM	24.1 ± 2.2	26.3 ± 3.0	22.8 ± 4.0	23.3 ± 1.9	26.0 ± 4.9	24.5 ± 6.2
5 μM	31.9 ± 3.8	36.8 ± 2.1	33.3 ± 3.8	36.0 ± 3.6	36.8 ± 5.9	23.7 ± 4.5
Thrombin 1 U/ml	12.9 ± 4.9	12.9 ± 4.5	13.5 ± 5.3	12.3 ± 4.8	14.1 ± 4.8	27.0 ± 12.9
2 U/ml	28.4 ± 4.0	35.6 ± 2.9	40.4 ± 4.4	26.4 ± 2.6	33.6 ± 6.4	37.8 ± 5.0
Thromboxane B_2 production (ng/ml PRP)						
ADP 2 μM	3.2 ± 0.2	3.0 ± 0.4	2.8 ± 0.2	2.6 ± 0.3	3.4 ± 1.1	2.6 ± 0.3
5 μM	3.8 ± 0.3	3.2 ± 0.2	3.7 ± 0.2	3.1 ± 0.5	3.6 ± 0.7	2.7 ± 0.5
Thrombin 1 U/ml	2.8 ± 0.2	2.6 ± 0.2	2.4 ± 0.1	2.3 ± 0.2	4.1 ± 2.1	2.8 ± 0.3
2 U/ml	6.6 ± 1.2	12.5 ± 5.1	6.4 ± 1.3	8.4 ± 1.4	11.9 ± 2.2	7.0 ± 1.8
Arachidonic acid 6.4 mM	1440 ± 141	1406 ± 159	1671 ± 204	1413 ± 85	1202 ± 67	1365
Platelet-poor plasma	2.9 ± 0.3	2.2 ± 0.2	2.4 ± 0.1	1.9 ± 0.1	2.7 ± 0.7	1.8 ± 0.2

Mean ± SEM.

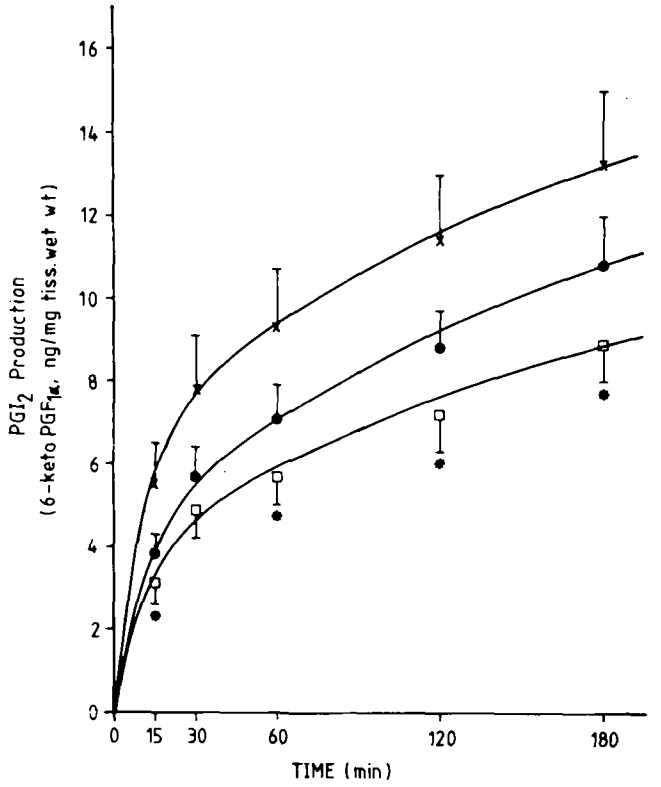

FIG. 1. Time course of prostacyclin production by rings of abdominal aorta from rats fed diets containing 10% (x), 30% (•) or 50% (□) energy from butter (ng 6-keto $PGF_{1\alpha}$/mg tissue wet weight, mean ± SEM).

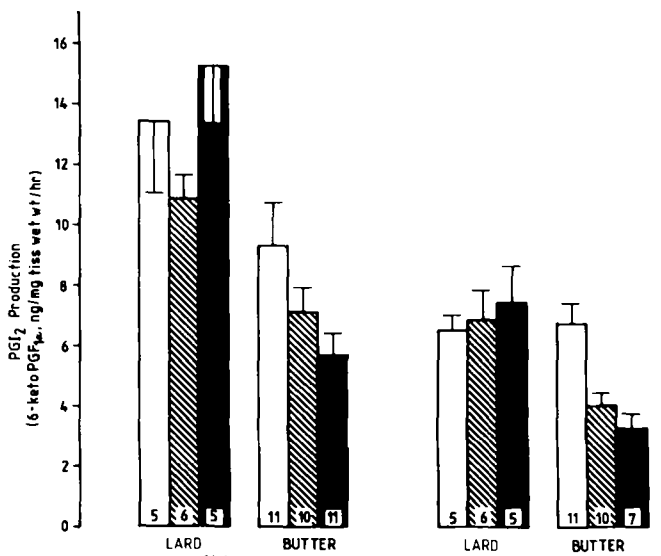

FIG. 2. Prostacyclin production by rings of abdominal aorta and mesenteric artery from rats fed diets containing 10% (open bars), 30% (hatched bars) or 50% (black bars) energy from butter or lard.

Statistics:	Abdominal aorta	Mesenteric artery
10% vs 30%	butter n.s., lard n.s.	butter $p < 0.005$, lard n.s.
10% vs 50%	butter $p < 0.05$, lard n.s.	butter $p < 0.001$, lard n.s.
30% vs 50%	butter n.s., lard n.s.	butter n.s., lard n.s.

of incubation in order to summarize the data in a single figure (Fig. 2). In both the mesenteric artery and the abdominal aorta, the PGI_2 production decreased in a dose-dependent manner as the proportion of butter in the diet rose from 10% to 50% energy. In contrast, lard supplementation had no detectable effect on PGI_2 production in either artery.

The effects of butter-enrichment on the fatty acid composition of plasma phospholipids are shown in Table 4. Increasing the butter content of the diet was associated with striking concentration-dependent changes in the proportions of the eicosanoid fatty acids: AA fell ($p < 0.001$), EPA rose ($p < 0.001$) and eicosatrienoic acid (20:3 n-9) rose ($p < 0.001$). In contrast, lard-enrichment had no effect on the plasma phospholipid fatty acid composition. None of the lard diets differed from the 10% butter diet or from each other (Table 5).

The effect of increasing the butter content of the diet on the fatty acid composition of aortic phospholipids is presented in Table 6. The major changes were in the proportions of the eicosanoid fatty acids that were similar but less pronounced than those in the plasma phospholipids. The proportion of AA fell but that of EPA and 20:3 n-9 rose as the butter content of the diet increased. Enrichment of the diet with lard had no effect on the fatty acid composition of aortic phospholipids.

The concentration of phospholipid in plasma fell dose-dependently on the butter-enriched diets: 10% = 54.8 ± 3.0, 20% = 50.0 ± 2.1, 50% = 41.6 ± 2.6 mg phospholipid fatty acid / 100 ml plasma (50% < 10%, $p<0.01$; 50% < 30%, $p<0.05$). The concentration of phospholipids in aorta and liver was not affected by increasing the butter content of the diet. Increasing the proportion of dietary fat as lard did not affect the phospholipid concentration in plasma, aorta or liver. The data are not shown but in all three lard-enriched diets, it was essentially the same as that in the 10% butter diet.

In view of the changes in aortic lipid composition and the marked reduction in aortic prostacyclin production on the butter-enriched diets, we considered the possibility that the synthesis and/or release of EDRF from the endothelium also could have been influenced. To test for this possibility, aortic smooth muscle reactivity and the release of EDRF were determined. There was a small but significant difference in the contraction response to submaximal concentrations of phenylephrine (1 μM) between the 10% butter diet (2.73 ± 0.08 g, n=6) and the 50% butter diet (3.09 ± 0.07 g, n=6, $p<0.02$) (Fig. 3). In the 50% butter diet, the maximum relaxation to acetylcholine was significantly less (1.08 g) than that on the 10% butter diet (1.62 g). In percentage terms, these relaxations were 34.9% and 59.3%, respectively. The sensitivity EC_{50} of the two diet groups to acetylcholine was similar (EC_{50}: 10% butter diet = 0.21 μM, 50% butter diet = 0.33 μM).

DISCUSSION

The major finding in this study was that increasing the level of butter in the diet resulted in a progressive reduction in PGI_2 production in aorta and mesenteric

TABLE 4

The Effects of Butter-enrichment on the Fatty Acid Composition of Plasma Phospholipids

Fatty acid	Percentage distribution of fatty acids (Mean ± SEM) Diet 10% n=11	30% n=9	50% n=10	Statistical comparisons 10% vs 30%	10% vs 50%	30% vs 50%
16:1	24.6 ± 0.6	23.1 ± 1.0	22.6 ± 0.7	—	—	—
16:1	1.4 ± 0.2	1.1 ± 0.2	0.9 ± 0.2	—	—	—
18:0	20.3 ± 0.6	23.2 ± 0.9	23.2 ± 0.7	0.05	0.01	—
18:1	9.4 ± 0.2	10.8 ± 0.7	13.5 ± 0.9	—	0.001	0.05
18:2n-6	13.3 ± 0.3	13.6 ± 0.4	11.9 ± 0.4	—	0.05	0.05
20:3n-9	0.7 ± 0.06	1.2 ± 0.09	1.9 ± 0.1	0.001	0.001	0.001
20:3n-6	0.9 ± 0.08	1.2 ± 0.09	1.6 ± 0.05	0.05	0.001	0.01
20:4n-6	23.4 ± 0.4	17.3 ± 0.9	12.3 ± 0.7	0.001	0.001	0.001
20:5n-3	0.2 ± 0.03	0.9 ± 0.08	2.6 ± 0.3	0.001	0.001	0.001
22:5n-3	0.6 ± 0.06	0.9 ± 0.1	1.5 ± 0.09	—	0.001	0.01
22:6n-3	5.2 ± 0.3	6.7 ± 0.4	8.0 ± 0.4	0.01	0.001	0.05

TABLE 5

The Effects of Lard-enrichment on the Fatty Acid Composition of Plasma Phospholipids

Fatty acid	Percentage distribution of fatty acids (mean ± sem) Diet 10% n = 4	30% n = 4	50% n = 4
16:0	24.1 ± 1.9	21.3 ± 2.0	23.5 ± 2.2
16:1	1.0 ± 0.2	0.5 ± 0.08	0.4 ± 0.2
18:0	18.4 ± 1.5	21.2 ± 2.2	20.0 ± 0.6
18:1	8.2 ± 0.5	5.9 ± 1.0	9.9 ± 0.8
18:2n-6	12.8 ± 0.7	11.4 ± 0.7	13.7 ± 1.4
20:3n-9	0.5 ± 0.07	0.5 ± 0.03	0.5 ± 0.05
20:3n-6	0.5 ± 0.08	0.7 ± 0.03	0.9 ± 0.09
20:4n-6	29.6 ± 1.9	31.9 ± 2.8	25.7 ± 1.8
20:5n-3	0.1 ± 0.02	0.3 ± 0.09	0.6 ± 0.2
22:5n-3	0.5 ± 0.07	0.7 ± 0.1	0.7 ± 0.2
22:6n-3	4.4 ± 0.7	5.6 ± 0.8	4.1 ± 0.7

artery in vitro. This effect on arterial PGI_2 production was accompanied by profound changes in the fatty acid composition of plasma phospholipids which were also evident but less marked in aortic phospholipids. The proportion of AA fell markedly as the level of butter in the diet increased, whereas the proportion of EPA and eicosatrienoic acid (20:3n-9) rose. The reductions in arterial PGI_2 production can be explained in terms of these fatty acid changes: the fall in the concentration of its precursor (AA) in arterial wall phospholipids, the rise in the concentration of EPA (an inhibitor of cyclooxygenase [15]), or a combination of both.

Substitution of lard for butter as the major source of fat in the diet did not result in any changes in fatty acid composition of plasma, aortic or liver phospholipids, nor was it associated with any reduction in arterial PGI_2 production. This latter result provides good evidence that the progressive reduction in arterial PGI_2 production in the butter–fed rats was not due to the increasing proportion of dietary fat per se. It also raises a number of questions.

Were the different responses to enrichment of the diet with butter or lard due to the different P/S ratios of the diets? The P/S ratios of the 10% fat diets were quite similar regardless of whether lard or butter were used (0.54 and 0.56, respectively). However, as the fat content of the diet was increased to 30% and 50% the P/S ratio fell much more with butter (0.10 and 0.05) than it did with lard (0.24 and 0.19). It is possible that both the concentrations and the relative proportions of particular polyunsaturated fatty acids (PUFA) are critical factors in determining the changes in phospholipid fatty acid composition and the consequent physiological responses.

What was the source of the increased concentration of EPA in plasma and tissue phospholipids of the butter–fed rats? It is unlikely to have come from the butter itself because the only PUFA we could detect in butter were linoleic and linolenic acids. A more likely explanation could be that the ratio of linoleic acid/linolenic acids was much lower in the butter than the lard diets. In the 50% fat diets, this ratio was 9:1 with lard and only 2:1 with butter. Since linolenic acid is a more effective competitor for the desaturase enzymes than linoleic acid in the rat (16), the lower linoleate/linolenate ratio in the butter diets may have facilitated the synthesis of EPA at the expense of AA.

Studies on the effects of different types of dietary fat on arterial PGI_2 production in the rat and rabbit have yielded conflicting results (17–20). Not unexpectedly, essential fatty acid deficiency reduced aortic PGI_2 production (17). Diets enriched with linoleic acid (the precursor of AA) have not been associated with enhanced PGI_2 production but rather a reduction (18,19). Lard supplementation has been reported not to change (20) or to increase (19) basal aortic prostacy-

TABLE 6

The Effects of Butter-enrichment on the Fatty Acid Composition of Aortic Phospholipids

Percentage distribution of fatty acids (Mean ± sem)				Statistical comparisons		
Fatty acid	Diet					
	10% n=9	30% n=8	50% n=9	10% vs 30%	10% vs 50%	30% vs 50%
16:0	23.1 ± 0.8	22.7 ± 0.9	23.1 ± 1.1	—	—	—
16:1	2.9 ± 0.4	2.5 ± 0.5	3.0 ± 0.7	—	—	—
18:0	17.4 ± 0.9	18.5 ± 1.1	16.7 ± 1.4	—	—	—
18:1	15.0 ± 0.5	16.1 ± 0.3	16.7 ± 0.3	—	0.05	—
18:2n-6	6.0 ± 0.3	5.6 ± 0.2	5.2 ± 0.3	—	—	—
20:3n-9	1.5 ± 0.09	1.4 ± 0.2	2.0 ± 0.1	—	0.01	0.01
20:3n-6	1.8 ± 0.07	1.9 ± 0.09	1.6 ± 0.07	—	—	—
20:4n-6	23.9 ± 0.5	22.4 ± 0.8	21.1 ± 0.8	—	0.01	—
20:5n-3	0.5 ± 0.05	0.9 ± 0.2	2.2 ± 0.1	0.01	0.001	0.001
22:4n-6	3.0 ± 0.2	2.7 ± 0.2	1.9 ± 0.1	—	0.001	0.001
22:5n-6	0.8 ± 0.04	0.8 ± 0.09	0.5 ± 0.03	—	0.001	0.001
22:5n-3	1.0 ± 0.07	1.4 ± 0.1	2.2 ± 0.1	0.01	0.001	0.001
22:6n-3	3.1 ± 0.1	3.1 ± 0.2	3.8 ± 0.3	—	—	—

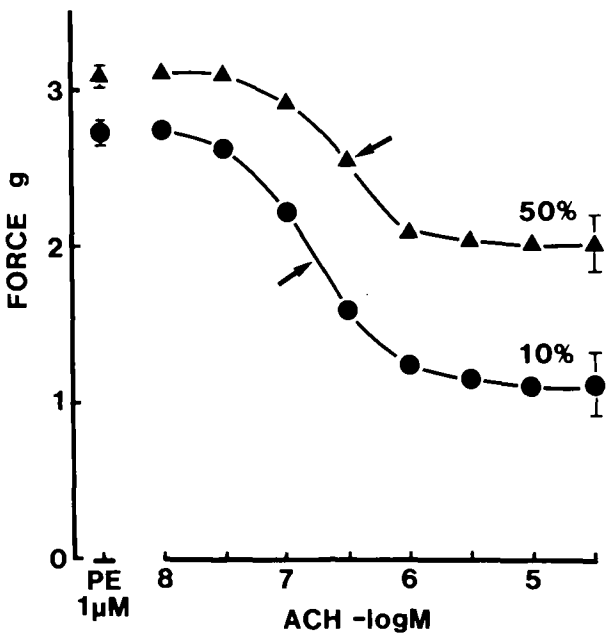

FIG. 3. Average acetylcholine-relaxation curves in six rings precontracted by phenylephrine (1 μM). The isometric force (g) is calculated as the change in the active force after the initial stretch-force procedure. The aortas were removed from rats on the 10% butter diet (•) or 50% butter diet (▲). Error bars shown are ± 1 SEM.
PE, phenylephrine 1 μM. ACH, acetylcholine. EC_{50} values are indicated by arrow.

clin production and to stimulate PGI_2 synthesis from exogenous AA (20). In contrast to the results of the present study, Galli and coworkers reported increased aortic PGI_2 production in rabbits red diets containing 25% energy as butter (19). It is possible that the variable effects of dietary saturated fat on aortic prostacyclin production in the rat that have been reported in the literature (18–21) also may be due to variations in the fatty acid composition of the rat chows used for the control diets. We have observed wide variations in plasma phospholipid fatty acid composition between rats fed different batches of chow (purchased from the same supplier), which is the reason we now prepare all diets in the laboratory. In the few instances in which effects of the diets on the fatty acid composition of aortic phospholipids have been measured, any changes in PGI_2 production have been mirrored by changes in aortic phospholipid AA content. When AA content increased (19,20) so did PGI_2 production and vice versa (17,22). This highlights the importance of measuring effects of diets on phospholipid fatty acid composition. In this study, changes in plasma phospholipid fatty acid composition were similar but more pronounced than those in the aorta.

Sinzinger and coworkers have suggested that the relative resistance of rats to the dietary induction of atherosclerosis may be related, at least in part, to their particularly high capacity for arterial PGI_2 synthesis relative to man, rabbit and a series of other animals that were tested (23). This characteristic, in turn, may be related to the high levels of AA in rat arterial phospholipids (20). It also is possible that the negligible thromboxane production by platelets after stimulation with ADP and thrombin also could contribute to this resistance to vascular disease.

Although the butter-enriched diets had clear-cut effects on the fatty acid composition of plasma and tissue phospholipids that correlated well with reductions in arterial PGI_2 production in vitro, there were no significant effects on ADP or thrombin-induced platelet aggregation. It is possible that the diet period (three weeks) was not sufficiently long for any such effects to be evident (24). Longer feeding periods

should clarify this question. Similarly, TXA_2 production by platelets in vitro was not affected by increasing levels of either type of fat in the diet. Indeed, TXA_2 production by platelets after stimulation with ADP and thrombin generally was insignificant, i.e. not significantly greater than that generated by the PPP "blank."

These results also may shed light on the observation of Morita and coworkers that dietary supplementation with EPA inhibited platelet aggregation in rats only when the background diet had been enriched with butter (25). The mechanism by which butter–enrichment unmasked this effect of EPA may be related to effects on the fatty acid composition of platelets. Presumably, the butter, being particularly low in PUFA and with an unusually low linoleate/linolenate ratio. would have been associated with reduced AA synthesis and thereby have facilitated the incorporation of the EPA dietary supplement into platelet phospholipids, resulting in reduced platelet aggregation. The data from the present study suggest that there is enrichment of tissue phospholipids with EPA even in the absence of supplementation.

In view of the changes in aortic phospholipid fatty acid composition on the butter–enriched diet, the possibility that alterations in membrane lipids could affect the synthesis and/or the release of EDRF from the endothelial cell or even the membrane receptor for the EDRF releasing agonist such as acetylcholine was considered. A very recent paper (26) has identified EDRF as nitric oxide (NO), at least in bovine endothelial cells stimulated by bradykinin. EDRF is a highly labile (27) nonprostanoid factor released from endothelial cells that has a powerful vasodilatory action on the underlying smooth muscle in response to some vasoactive substances such as acetylcholine, substance P, bradykinin and ATP (28). Because in the rat aorta (as in other species) the relaxation response to acetylcholine completely is endothelium-dependent (29), the reactivity to acetylcholine can be used to assess the entire process from EDRF release to smooth muscle relaxation. The 50% butter diet attenuated the magnitude of the EDRF-mediated response range suggesting either a reduction in the EDRF synthesis or release, or in smooth muscle responsiveness. Our experiments cannot distinguish between these possibilities. The sensitivity (ED50) was altered little, suggesting that the acetylcholine receptor coupling probably was unaffected by the dietary change. These changes to the EDRF activity may have been related to the marked changes in the PUFA composition of aortic phospholipids. However, the marked reduction in prostacyclin release measured in the separate series of experiments could not have been responsible for these changes in EDRF response since indomethacin was present throughout these latter experiments.

In conclusion, these results provide strong evidence that in the rat (i) dietary saturated fat in the form of butter can reduce the concentration of AA in plasma and tissue phospholipids, (ii) vascular PGI_2 production appears to be modulated directly by the level of AA in arterial wall phospholipids and/or inversely by the level of EPA, (iii) changes in plasma phospholipid fatty acid composition appear to be an accurate reflection of changes in arterial wall phospholipid fatty acid composition, (iv) these changes in aortic phospholipid fatty acid composition and PGI_2 production were accompanied by a reduction in smooth muscle relaxation to EDRF. Finally, butter-supplementation may provide an ideal model in which to examine the effects of particular PUFA supplements on vascular PGI_2 production in the rat.

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REFERENCES

1. Blackburn, H. (1983) *Prev. Med. 12*, 2–10.
2. Shekelle, R.B., Shryock, A.M., Paul, O., Lepper, M., Stamler, J., Lin, S., and Raynor, W.J. (1981) *New. Engl. J. Med. 304*, 65–70.
3. Renaud, S. (1974) *Thromb. Res.* (Suppl 1) *4*, 25–35.
4. McGregor, L., Morazain, R., and Renaud, S. (1980) *Lab. Invest. 43*, 438–442.
5. Lagarde, M., Burtin, M., Rigaud, M., Sprecher, H., Dechavanne, M., and Renaud, S. (1983) *Lipids 18*, 291–294.
6. Collins, F.D., Sinclair, A.J., Royle, J.P., Coats, D.A., Maynard, A.T., and Leonard, R.F. (1971) *Nutr. Metabol. 13*, 150–167.
7. Bieri, J.G., Stoews, G.S., and Briggs, G.M. (1977) *J. Nutr. 107*, 1340–1348.
8. Best, L., McGuire, M., Martin, T.J., Preston, E., and Russell, R.G.G. (1979) *Biochim. Biophys. Acta 583*, 344–351.
9. Nolan, R.D., Dusting, G.J., and Martin, T.J. (1981) *Biochem. Pharmacol. 30*, 2121–2125.
10. Angus, J.A., Cocks, T.M., and Satoh, K., *Br. J. Pharmacol.*, in press.
11. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497–509.
12. Sinclair, A.J., O'Dea, K., and Naughton, J.M. (1983) *Lipids 18*, 877–881.
13. Bloom, W.L. (1962) *Arch. Intern. Med. 109*, 26–32.
14. Bloom, W.L., and Azar, G.J. (1963) *Arch. Intern. Med. 112*, 333–337.
15. Needleman, P., Raz, A., Minkes, M.S., Ferrendelli, J.A., and Sprecher, H. (1979) *Proc. Natl. Acad. Sci. USA 79*, 944–948.
16. Brenner, R.R. (1974) *Mol. Cell Biochem. 3*, 41–52.
17. Sakr, H.M., and Dunham, E.W. (1985) *Prost. Leuk. Med. 18*, 183–192.
18. de Deckere, E.A.M., Nugteren, D.H., and ten Hoor, F. (1979) *Prostaglandins 17*, 947–955.
19. Galli, C., Agradi, E., Petrone, A., and Tremoli, E. (1981) *Lipids 16*, 165–172.
20. Wey, H.E., Ziller, S., and Subbiah, M.T.R. (1983) *Prost. Leuk. Med. 12*, 169–174.
21. Abeywardena, M.Y., McLennan, P.L., and Charnock, J.S. (1986) *Prost. Leuk. Med. 25*, 209–221.
22. Cox, J.W., Rutechi, G.W., Francisco, L.L., and Ferris, T.F. (1982) *Circ. Res. 51*, 694–702.
23. Sinzinger, H., Clopath, P., Silberbauer, K., and Winter, M. (1980) *Experimentia 36* 321–323.
24. Davenas, E., Ciavatti, M., Nordoy, A., and Renaud, S. (1984) *Biochim. Biophys. Acta 793*, 278–286.
25. Morita, I., Kameshina, N., Takahashi, R., Orimo, H., and Murota, S. (1984) *Prost. Leuk. Med. 14*, 123–129.
26. Palmer, R.M.J., Ferrige, A.G., and Moncada, S. (1987) *Nature 327*, 524–526.

27. Cocks, T.M., Angus, J.A., Campbell, J.H., and Campbell, G.R. (1985) *J. Cell. Physiol. 123*, 310–320.
28. Furchgott, R.F., and Zawadski. J.V. (1980) *Nature 288*, 373–376.
29. Furchgott, R.F. (1984) *Ann. Rev. Pharmacol. Toxicol. 24*, 175–197.

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Lipogenic Enzyme Activities in Primary Cultures of Adult Mouse Hepatocytes

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The effects of various unsaturated fatty acids such as oleic (18:1n-9), linoleic (18:2n-6) and arachidonic (20:4n-6) on the activities of fatty acid synthetase (FAS), malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) all were determined in primary cultures of mouse hepatocytes. Activities of FAS and ME were found to decrease with time in culture regardless of whether hepatocyte donors were fed diets containing polyunsaturated fatty acid-free hydrogenated cottonseed oil (HCTO) or corn oil (CO). On the other hand, while G6PDH activity also declined in cultured hepatocytes obtained from HCTO-fed mice, the activity of this enzyme increased in cells cultured from CO-fed mice. 6PGDH activity was found to increase in hepatocytes obtained from both diet groups. Neither 18:2 nor 20:4 when added to media could alter FAS or ME activities compared with those observed with either 18:1-containing or fatty acid-free media. Since lactic dehydrogenase activity and the rate of incorporation of [^{3}H] leucine into FAS protein were unaltered with time in hepatocyte cultures, the decreased activities of FAS and ME cannot be attributed to a loss in cell viability during culture but rather appear to be specific for those enzymes which respond to diet hormones in vivo. Examination of the fatty acid contents of the cells after the culture period showed that the values for the ratios of 16:0/16:1 and of 18:0/18:1 were elevated when either 18:2 or 20:4 was added to the medium even though there was no evidence for elongation of the added 18:2 or for 20:4 being converted to 22:4. This result suggest that Δ9-desaturase activity was inhibited by these polyunsaturated fatty acids and that conversion of 18:2 to 20:4 was not required for such action.

The rate of synthesis determined by the relative rate of incorporation of [^{3}H]leucine into FAS was two to five times higher in hepatocytes prepared from mice fed the HCTO diet than in hepatocytes from mice fed the CO diet. We have concluded that the mechanisms for long-term regulation may not be contained entirely within the liver.

Lipids 23, 242–247 (1988).

Previously, we examined the influence of 18- and 20-carbon-chain-length fatty acids on the mechanisms involved with lipogenesis in mouse liver (1,2) as part of our continuing investigation of the long-term control of this process. Such control is accomplished through the regulation of the cellular contents of specific enzymes (3–5) rather than through allosteric mechanisms. In those studies, we showed that linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), columbinic acid (5-*trans*,9-*cis*,12-*cis*-18:3n-6) and arachidonic acid (20:4n-6), either in the diet or administered by injection, reduced the levels of fatty acid synthetase (FAS), malic enzyme (ME) and ATP-citrate lyase in the liver when compared with saturated or monounsaturated fatty acids, neither of which had any effect. If the mechanisms involved in the polyunsaturated fat inhibition of lipogenesis are contained entirely within the liver, experiments with isolated cells might reveal these mechanisms. Hence, in this study we employed primary hepatocyte cultures since these cells maintain for several days many of the morphological and biochemical characteristics observed in situ (6–12). We examined in the hepatocyte cultures the influence of the nature of the dietary fat fed to donor mice on cellular fatty acid composition, on FAS, ME, glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH) and lactic dehydrogenase (LDH) activities, and on the relative rate of synthesis of the enzyme FAS. We also examined the effect of pure fatty acids (18:1n-9, 18:2n-6 and 20:4n-6) when added to the media during culture on the levels of FAS and ME.

MATERIALS AND METHODS

Animals and diets. BALB/c mice were used throughout. They originally were obtained from Simonsen Labs (Gilroy, CA) and currently are bred and maintained in our vivarium. The mice were housed on pine shavings in plastic cages and were kept in a temperature-controlled room (22–23 C) on a 12-hr light, 12-hr dark cycle. Purina mouse chow #5015 and tap water were available ad libitum before use. The chow diet contained 11% fat, of which 44% of the constituent fatty acids was linoleate. Mice employed as liver donors were allowed to eat the semipurified diets ad libitum for one week. These isocaloric diets were prepared in our laboratory and contained either 10% hydrogenated cottonseed oil (HCTO) or 10% corn oil (CO). The composition of the semipurified diets (13) and the fatty acid composition of the two fats (14) have been reported. Corn oil contains about 60% of its constituent fatty acids as linoleic acid, whereas HCTO has less than 0.5%.

Isolation of hepatocytes and culture conditions. Hepatocytes were isolated from adult BALB/c mice following perfusion of the liver in situ according to the procedure described by Dougherty et al. (15). The portal vein was cannulated and sterile Hank's Balanced Salt Solution (HBSS) containing 0.19 mg/ml EGTA at 37 C was pumped through the liver for seven min at a rate of 7.2 ml/min. This was followed by perfusion with HBSS containing 0.5 mg/ml collagenase (Sigma Type IV) for an additional 24 min at the same

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Abbreviations: CO, cottonseed oil; FAS, fatty acid synthetase; G6PDH, glucose-6-phosphate dehydrogenase; HBSS, Hank's balanced salt solution; HCTO, hydrogenated cottonseed oil; ME, malic enzyme; 6PGDH, 6-phosphogluconate dehydrogenase.

rate. The liver was removed, minced and filtered through 64 μM mesh nylon bolting cloth. The cells obtained were washed three times with perfusion buffer without enzyme, and their viability determined by trypan blue exclusion. Only preparations judged to have a viability of 95% or greater were used. The hepatocytes then were suspended in culture medium at a concentration of 0.9×10^6 cells per ml and aliquots (3 ml) added to each 60 mm Primaria tissue culture dish (Falcon). The cells were incubated at 37 C in an atmosphere of air/CO_2 (95%:5%).

The basic culture medium was Waymouth's medium (MB 752/1) supplemented, per liter, with 11.2 mg L-alanine, 24 mg L-asparagine, 12.8 mg L-serine, 2.24 g $NaHCO_3$, 100 mg streptomycin sulfate, 84 mg gentamicin sulfate, 61 mg (100,000 units) penicillin, 1 mg insulin and 550 mg sodium pyruvate. The plating medium contained the basic culture medium to which 10% fetal bovine serum was added. After allowing the cells to attach to the plates for four hr, the plating medium was replaced with basic culture medium containing 1×10^{-6} M triiodothyronine, 1×10^{-6} M hydrocortisone, 1×10^{-7} M insulin and 0.5% BSA either fatty acid–free or with 0.34 mM of either oleate, linoleate or arachidonate. The medium was replaced every 24 hr thereafter with fresh basic medium containing the appropriate additions.

Enzyme protein synthesis. The synthesis of fatty acid synthetase was determined directly by measuring the rate of incorporation of L-[4,5,^{3}H]leucine into fatty acid synthetase relative to the rate of incorporation into the total soluble protein of the cytosol (1,16). For this purpose, hepatocyte cultures were incubated with basic medium containing 10 μc L-[4,5^{3}H]leucine per ml for two hr at 37 C. The hepatocytes were washed four times with cold 0.25 M sucrose, homogenized at 0–4 C in a Potter-Elvehjem tissue grinder with 0.7 ml 0.25 M sucrose, centrifuged for one hr at 100,000 × g, and the clear supernatent (cytosol) removed. Methods for the isolation of total soluble protein, the immunoprecipitation of fatty acid synthetase and the assay of radioactivity have been described (1).

Analytical procedures. Procedures for the saponification of lipids, the extraction of fatty acids, preparation of methyl esters and the determination of fatty acid composition by gas liquid chromatography were described previously (17). The fatty acid methyl esters were identified by comparison of retention times

TABLE 1

Effect of Dietary Fat Fed to Hepatocyte Donors and Time in Culture, on Specific Enzyme Activities[a]

Expt. series	Dietary fat (10%)	Enzyme	Added fatty acid	Time in culture (hr) 4	22	70
1	HCTO	FAS (3)	18:1	40.9 ± 7.7	30.5 ± 6.3	10.4 ± 1.3
		ME (3)	18:1	264.5 ± 11.2	142.9 ± 18.5	81.4 ± 12.5
		G6PDH (3)	18:1	198.1 ± 14.2	137.6 ± 13.4	64.2 ± 13.3
		6PGDH (3)	18:1	31.6 ± 2.1	29.3 ± 8.8	50.3 ± 5.5[b]
	HCTO	FAS (3)	18:2	—	30.7 ± 50	6.6 ± 3.1
		ME (3)	18:2	—	182.7 ± 23.5	165.4 ± 12.7
		G6PDH (3)	18:2	—	130.7 ± 7.0	81.3 ± 9.7
		6PGDH (3)	18:2	—	32.1 ± 1.3	69.2 ± 12.2[c]
	HCTO	FAS (3)	20:4	—	27.0 ± 5.5	8.9 ± 4.3
		ME (3)	20:4	—	164.9 ± 23.2	43.0 ± 2.4
		G6PDH (3)	20:4	—	114.3 ± 8.0	64.9 ± 14.1
		6PGDH (3)	20:4	—	29.3 ± 1.5	53.8 ± 6.3[c]
2	HCTO	FAS (6)	18:1	41.3 ± 8.5	25.1 ± 7.1	11.4 ± 3.3
		FAS (6)	20:4	—	26.8 ± 11.1	8.9 ± 3.3
		ME (6)	18:1	248.5 ± 28.4	147.5 ± 25.1	76.9 ± 10.3
		ME (6)	20:4	—	153.8 ± 40.9	75.8 ± 27.1
3	HCTO	FAS (6)	—	38.7 ± 6.7[b]	21.0 ± 5.8[b]	9.1 ± 1.9[b]
		ME (7)	—	231.2 ± 42.6[b]	150.8 ± 27.4[b]	81.6 ± 9.8[b]
		G6PDH (3)	—	170.6 ± 40.0[b]	121.2 ± 14.7[b]	65.8 ± 12.9[b]
		6PGDH (3)	—	32.3 ± 3.3[b]	32.0 ± 5.6[b]	56.6 ± 9.8[b,c]
		LDH (6)	—	1958 ± 599	2086 ± 629	2071 ± 209
	CO	FAS (4)	—	18.3 ± 6.2	10.0 ± 2.7	5.4 ± 2.4
		ME (3)	—	71.4 ± 4.0	56.3 ± 16.4	30.2 ± 8.2
		G6PDH (3)	—	5.2 ± 3.7	9.1 ± 3.3	9.4 ± 3.4[c]
		6PGDH (3)	—	12.4 ± 5.6	15.2 ± 6.6	29.9 ± 8.8[c]
		LDH (6)	—	1929 ± 481	2148 ± 601	2061 ± 573

[a]Results are presented as mean ± S.D. of enzyme specific activity (nmol substrate converted to product/min per mg cytosol protein). Figures in parentheses are the number of experiments with different hepatocyte preparations; four cultures were analyzed per time period in each experiment. The concentration of 18:1, 18:2 and 20:4 added to the medium after the four-hr plating period was 0.34 mM.
[b]Significantly different from corresponding CO values at $p < 0.05$.
[c]Significantly greater than corresponding values for 4- or 22-hr culture at $p < 0.05$.

with those of authentic standards obtained from NuCheck-Prep, Inc. (Elysian, MN).

The assay procedures for fatty acid synthetase (18), malic enzyme (E.C. 1.1.1.40) (19), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) (20), 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44) (21) and lactic dehydrogenase (E.C. 1.1.1.27) with lactate as substrate (22) have been reported. Units of enzyme activity are given as nmol pyridine nucleotide oxidized or reduced per minute at 30 C under the conditions of the specific assay. Protein was determined by the method of Lowry et al. (23).

RESULTS

We as well as others (6,10–12) have found that during culture, the activities of several hepatic lipogenic enzymes decrease with time (Table 1). Various culture modifications were made in attempts to maintain the activities of the lipogenic enzymes at the levels observed in freshly isolated hepatocytes. Although insulin (10^{-7} M) and pryruvate (5 mM) slowed the decline in the activities of fatty acid synthetase and malic enzyme, the loss of enzyme activity could not be prevented completely. The compounds studied included hydrocortisone or dexamethasone, thyroxine, triiodothyronine, glucagon, α-tocopherol, testosterone, 17-β-estradiol and L-ascorbic acid. Even under the best conditions used, from 60–80% of the enzyme activity observed for FAS and ME in the freshly isolated hepatocytes was lost during the subsequent 70–hr incubation period (Table 1).

This rate of decline in FAS and ME activities observed during culture was similar for hepatocytes isolated from livers of mice fed either the HCTO or the CO diet (Table 1). The alterations in G6PDH and 6PGDH activities differed from those observed with FAS and ME. The activity of G6PDH declined in hepatocytes prepared from HCTO–fed mice and increased significantly in those from CO–fed mice. The activity of 6PGDH consistently increased after the first 22 hr of culture in hepatocytes from both HCTO–fed and CO–fed mice. However, the differences in activities observed for all four enzymes in freshly isolated hepatocytes between HCTO– and CO–fed hosts were maintained in culture and were significant at all three time periods examined. The magnitude of the difference in activities, as well as in the rates of decline in activity for both FAS and ME, were similar even if hepatocyte donors were fasted overnight before cell isolation (data not shown). On the other hand, neither the activity of a nonadaptive enzyme, lactic dehydrogenase (Table 1, Expt. Series 3), nor the rate of incorporation of [^{3}H]leucine into FAS protein (Table 2) declined during the culture period. Hence, the decrease in the activities of FAS and ME observed could not be attributed to a loss in hepatocyte viability during the culture period but rather appears to be specific for only certain adaptive enzymes.

No significant difference could be found in the FAS and ME activities between cultures without added fatty acids or those with either oleate, linoleate or arachidonate (Table 1). To determine whether the fatty acids added to the medium were taken up and metabolized by the cells, the fatty acid composition of hepatocyte lipids also was measured. When either oleate, linoleate or arachidonate was added to the medium as the sole fatty acid, each readily was taken up by the hepatocytes (Table 3). Freshly isolated hepatocytes from HCTO–fed mice contained 3.5% of the n-9 fatty acid 20:3, a product of 18:1 metabolism, but after 70 hrs of culture these cells contained only 0.6% (Table 3). Surprisingly, the proportion of 20:4 in the cells did not increase when 18:2 was added to the medium, although 22:4 levels were increased when

TABLE 2

Incorporation of [^{3}H]Leucine into Fatty Acid Synthetase (FAS) and Total Soluble Protein (TSP) of Cultured Hepatocytes Isolated From Either HCTO-fed or CO-Fed Mice[a]

Diet fed hepatocyte donor	Hr in culture	Added fatty acid	[^{3}H]Leucine incorporation into TSP (cpm/0.1 ml cytosol)	[^{3}H]Leucine incorporation into FAS (cpm/0.1 ml cytosol)	FAS/TSP ($\times 10^2$)
EXPT. 1					
HCTO	4	None	2912	378	12.8
	46	18:1	2750	263	9.6
	46	20:4	2092	249	11.9
EXPT. 2					
HCTO	22	None	13463 ± 168	885 ± 63	6.6 ± 0.5
	46	None	12733 ± 839	1056 ± 126	8.3 ± 0.5
CO	22	None	10841 ± 332	348 ± 47	3.2 ± 0.3
	46	None	11415 ± 694	191 ± 19	1.7 ± 0.3

[a]Cultures in Expt. 1 were incubated with 2.5 μCi and those in Expt. 2 with 10 μCi L-[4.5^{3}H]leucine as given in text. Results for experiment 1 are the values for two pooled cultures. Results for experiment 2 are given as the mean ± S.D. for two individual hepatocyte preparations. In each experiment, three separate cultures were used to obtain each data point.

TABLE 3

Fatty Acid Composition of Lipids from Hepatocytes Isolated from HCTO-fed Mice and Cultured in Media Containing either 18:1, 18:2 or 20:4 as the Sole Fatty Acid[a]

Cellular fatty acids	Freshly isolated cells	After 70 hr culture with		
		18:1	18:2	20:4
Saturated acids				
16:0	23.5 ± 0.1	19.6 ± 0.5	22.1 ± 0.5	27.1 ± 1.0
18:0	6.3 ± 0.2	7.3 ± 0.2	8.4 ± 0.3	8.9 ± 0.9
n-7 Acids				
16:1	5.6 ± 0.2	4.0 ± 0.2	2.4 ± 0.03	2.6 ± 0.01
n-9 Acids				
18:1	42.2 ± 1.2	62.8 ± 0.5	24.5 ± 0.5	24.4 ± 0.1
20:3	3.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.03	0.5 ± 0.01
n-6 Acids				
18:2	3.5 ± 0.01	0.8 ± 1.1	32.2 ± 0.5	1.4 ± 0.2
18:3	0.4 ± 0.01	0.3 ± 0.1	0.6 ± 0.1	1.1 ± 0.1
20:3	1.0 ± 0.1	0.2 ± 0.02	1.1 ± 0.1	0.9 ± 0.1
20:4	7.1 ± 0.1	1.5 ± 0.01	2.2 ± 0.3	22.9 ± 1.8
22:4	0.2 ± 0.1	0	0.2 ± 0.03	6.5 ± 0.2
Total n-6	12.0 ± 0	2.7 ± 0.2	36.7 ± 0.02	32.7 ± 2.3
Ratios				
16:0/16:1	4.24 ± 0.13	4.86 ± 0.13	9.20 ± 0.29	10.43 ± 0.42
18:0/18:1	0.15 ± 0	0.12 ± 0.01	0.34 ± 0.02	0.37 ± 0.04

[a]Results are presented as the mean percentage of total fatty acids ± SD for two experiments. Two cultures were analyzed for each condition in each experiment.

20:4 was the fatty acid added. The values for the ratios of 16:0/16:1 and of 18:0/18:1 were increased by the presence of either 18:2 or 20:4. Such findings suggest that desaturase activity was inhibited under these conditions, although elongation of 20:4 to 22:4 was not influenced.

The fatty acid composition of hepatocytes isolated from the livers of mice fed either the HCTO or the CO diet and cultured in fatty acid–free medium also was determined (data not shown). Under these conditions, a reduction in the percentage of polyunsaturated fatty acids and an increase in the proportion of oleate were observed. Based on experience with intact animals, this is exactly what one would expect to occur in the absence of exogenous PUFA.

The activities of lipogenic enzymes in cultures of hepatocytes from mice fed either the HCTO or the CO diet declined at the same rate. Since the time-course of change in enzyme activity is a function of the rate of degradation (24,25), the rate of degradation of FAS and ME appears to be unaffected by the previous nutritional state.

However, the data from the direct [^{3}H]leucine incorporation method (Table 2) show that the rate of synthesis of new FAS in hepatocytes is determined by the type of diet fed to the donor mice.

The relative rates of FAS synthesis were from two to five times higher for hepatocytes isolated from HCTO–fed mice than for those from CO–fed mice (Table 2, Expt. 2). The synthesis of FAS was maintained at a relatively constant rate during the 46–hr culture period in hepatocytes prepared from HCTO–fed mice. However, the relative rate of synthesis appeared to decline between 22 and 46 hr of culture in hepatocytes prepared from CO–fed mice. When either 18:1 or 20:4 was the sole fatty acid added to cultures of hepatocytes isolated from HCTO–fed mice, the values for the rates of incorporation of [^{3}H]leucine into FAS were the same (Table 2, Expt. 1). Indeed, the relative rates of synthesis of FAS in hepatocyte cultures in the presence of these fatty acids were comparable to those observed in the four hr cultures before adding the fatty acid–containing media. Thus, under these conditions, 20:4 did not depress the synthesis of FAS.

DISCUSSION

Studies with mice show that fats containing linoleate or other polyunsaturated fatty acids bring about a severe reduction in hepatic fatty acid synthesis (26) and depress the lipogenic response to refeeding (27). Allman and Gibson (26) and Muto and Gibson (28) suggested that polyunsaturated fatty acids are most effective in regulating hepatic lipogenesis in rats and mice. We found that a diet containing 15% safflower oil (linoleate content of more than 70%) reduced hepatic lipogenesis in both rats and mice (27). Musch et al. (29) have demonstrated that α-linolenate also decreased hepatic lipogenesis. Subsequently, we could show that dietary linoleate, α-linolenate, arachidonate and columbinate depressed hepatic lipogenesis and reduced the liver's content of key lipogenic enzymes (1,2). Hence, although it is well-established that di-and polyunsaturated fatty acids of both the n6 and n3 series depress hepatic lipogenesis, little is known about the mechanism and almost nothing is

known about the compound(s) that trigger the mechanism.

This study shows that FAS can be synthesized when the hepatocytes are cultured but the rates of synthesis always are higher when hepatocytes come from HCTO–fed than from CO–fed mice. In addition, the rates of decline in FAS and ME activities with time observed in these experiments with cultured hepatocytes suggest that the rate of enzyme degradation may not be affected by the previous nutritional state, since the time course of change in enzyme activity is a function of the rate of degradation (25,30) and not of synthesis. Hence, it would appear that the rates of synthesis in culture are not sufficient to maintain the enzyme content of these cells at a high level.

Since long-term regulation is accomplished through alterations in the rate of synthesis of key lipogenic enzymes, the decline in lipogenic enzymes in hepatocyte cultures indicates that important elements of the mechanism for long-term regulation either are missing or are not functional in such cultures. This would explain our finding that neither 18:2 nor 20:4 inhibit lipogenesis even though they readily are taken up and metabolized by the cells in these cultures.

Based on the values for the 16:0/16:1 and the 18:0/18:1 ratios of the total fatty acids from hepatocyte cultures, desaturase activity is inhibited by either 18:2 or 20:4. These results support the view that these polyunsaturated fatty acids enter the hepatocytes in sufficient quantities to affect some aspects of lipid metabolism and that regulation of the desaturase activity and the synthesis of lipogenic enzymes such as FAS and ME is accomplished through different mechanisms. Thus, it would appear that under certain conditions desaturase and fatty acid synthetase activities do not necessarily act in parallel as has been proposed by Jeffcoat and James (31).

Kelley et al. (12) reported that the prior dietary status of rats influences the activities of lipogenic enzymes in subsequent primary hepatocyte cultures. Hepatocytes obtained from rats fasted 24 hr and subsequently fed a high-carbohydrate diet for 48 hr yielded high values for liver FAS activity, which declined rapidly after the hepatocytes were cultured (12). Such results are consistent with our experience with mouse hepatocytes. However, these workers showed that when rats were fed a chow diet that contains a high PUFA content and thus similar to our corn oil-containing diet, FAS activity in such hepatocytes increased over two-fold during the culture period. We did not observe an increase in FAS activity in our primary mouse hepatocyte cultures.

Boogaerts et al. (32) have shown that hepatocytes in primary culture retain adaptive changes in lipogenesis and lipoprotein secretion induced in vivo by feeding rats a sucrose-enriched diet. The results of their study suggest that the increase in synthesis and secretion of triglyceride induced by media glucose is due to an increase in substrate availability rather than to long-term regulation that involves an increase in protein synthesis. Indeed, from this study with rats, Boogaerts et al. (32) suggested that the in vivo induction by dietary carbohydrate requires factors not contained within the isolated hepatocyte system. From the results presented here, we also have concluded that the long-term control of fatty acid synthesis exercised by certain dietary polyunsaturated fatty acids also does not occur entirely within the hepatocytes of the mouse.

Recently, Salati and Clarke (33) reported that saturated and polyunsaturated fatty acids equally suppress the hormonal induction of acetyl-CoA carboxylase in primary cultures of rat hepatocytes. Hence, the effect was nonspecific and therefore unrelated to the influence of dietary polyunsaturated fat on lipogenesis. These authors (33) suggested that an inhibitory factor derived from dietary polyunsaturated fatty acids might be produced in extrahepatic tissues and transported via the blood to the liver. Normal liver functions are known to be strongly influenced by the portal circulation (32,33–36), and thus the involvement of extrahepatic sites in hepatic adaptive processes appears feasible. Indeed, the liver appears to be the only tissue that responds to dietary polyunsaturated fat with a decrease in lipogenic capacity (17). Such additional evidence supports the hypothesis that substances in the portal circulation may play a role in long-term regulation of lipogenesis.

In conclusion, from the results presented here and by others (6–12,24,32,33,37), it would appear that certain adaptive changes caused by both carbohydrate and fat function only in intact animals and not in cultured hepatocytes. Therefore, it is possible that dietary constituents (i.e. carbohydrates and polyunsaturated fatty acids) induce in extrahepatic sites such as the gastrointestinal tract the production of regulatory compounds, which are transported via the portal vein to the liver where they may act on the hepatocyte apparatus responsible for the synthesis of the adaptive lipogenic enzymes. Thus, the polyunsaturated fatty acid-activated mechanisms for long-term regulation of fatty acid synthesis may not be contained entirely within the mouse hepatocyte. However, from the results obtained here it appears only those mechanisms that cause a suppression of desaturase activity by polyunsaturated fatty acids are still operational within the hepatocyte when cultured.

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REFERENCES

1. Schwartz, R.S. and Abraham, S. (1982) *Biochim. Biophys. Acta 711*, 316–326.
2. Abraham, S., Hillyard, L.A., Lin, C.Y., and Schwartz, R.S. (1983) *Lipids 18*, 820–829.
3. Korchak, H.M., and Masaro, E.J. (1962) *Biochim. Biophys. Acta 58*, 354–356.
4. Spencer, A.F., and Lowenstein, J.M. (1967) *Biochem. J. 103*, 342–346.
5. Guynn, R.W., Velosa, D., and Veech, R.L. (1972) *J. Biol. Chem. 247*, 7325–7331.

6. Spence, J.T., Pitot, H.C., and Zalitis, G. (1979) *J. Biol. Chem. 254,* 12169–12173.
7. Winberry, L., Nakayama, R., Wolfe, R., and Holten, D. (1980) *Biochem. Biophys. Res. Commun. 96,* 748–755.
8. Mariash, C.N., McSwigan, C.R., Towle, H.C., Schwartz, H.L., and Oppenheimer, J.H. (1981) *J. Clin. Invest. 68,* 1485–1490.
9. Nakamura, T., Yoshimoto, K., Aoyama, K., and Ichihara, A. (1982) *J. Biochem. 91,* 681–683.
10. Katz, N.R., and Giffhorn, S. (1983) *Biochem. J. 212,* 65–71.
11. Amatruda, J.M., Danaby, S.A., and Chang, C.L. (1983) *Biochem. J. 212,* 135–141.
12. Kelley, D.S., Nelson, G.J., and Hunt, J.E. (1986) *Biochem. J. 235,* 87–90.
13. Gabor, H., Hillyard, L.A., and Abraham, S. (1983) *J. Natl. Cancer Inst. 74,* 1299–1305.
14. Miyamoto-Tiaven, M.J., Hillyard, L.A., and Abraham, S. (1981) *J. Natl. Cancer Inst. 67,* 179–188.
15. Dougherty, K.K., Spilman, S.D., Green, C.E., Steward, A.R., and Byard, J.L. (1980) *Biochem. Pharmacol. 29,* 2117–2124.
16. Flick, P.K., Chen, J., and Vagelos, P.R. (1977) *J. Biol. Chem. 252,* 4242–4249.
17. Emken, E.A. Abraham, S., and Lin, C.Y., (1987) *Biochim. Biophys. Acta 919,* 111–121.
18. Smith, S., and Abraham, S. (1975) *Methods Enzymol. 35,* 65–74.
19. Ochoa, S. (1955) *Methods Enzymol. 1,* 739–753.
20. Kornberg, A., and Horecker, B.L. (1955) *Methods Enzymol. 1,* 323–326.
21. Horecker, B.L., and Smyrniotis, P.Z. (1955) *Methods Enzymol. 1,* 326–327.
22. Yoshida, A., and Freese, E. (1975) *Methods Enzymol. 41,* 304–309.
23. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
24. Stumpo, D.J., and Kletzien, R.E. (1985) *Biochem. J. 226,* 123–130.
25. Segal, H.L., and Kim, Y.S. (1963) *Proc. Natl. Acad. Sci. USA 50,* 912–916.
26. Allman, D.W., and Gibson, D.M. (1965) *J. Lipid Res. 6,* 51–62.
27. Bartley, J.C., and Abraham, S. (1972) *Biochim. Biophys. Acta 280,* 258–266.
28. Muto, Y., and Gibson, D.M. (1970) *Biochem. Biophys. Res. Commun. 38,* 9–15.
29. Musch, K., Ojakiam, M.A. and Williams, M.A. (1974) *Biochim. Biophys. Acta 337, 343–*348.
30. Schimke, R.T. (1975) *Methods Enzymol. 40,* 241–266.
31. Jeffcoat, R., and James, A.T. (1977) *Lipids 12,* 469–474.
32. Boogaerts, J.R., Malone-McNeal, M., Archambault-Schexnayder, J., and Davis, R.A. (1984) *Am. J. Physiol. 246,* 77–83.
33. Salati, L.M., and Clarke, S.D. (1986) *Arch. Biochem. Biophys. 246,* 82–89.
34. Patel, Y.C., Wheatley, T., and Ning, C. (1981) *Endocrinology 109,* 1943–1949.
35. Ruggere, M.D., and Patel, Y.C. (1984) *Endocrinology 115,* 244–248.
36. Burton, D.N., Collins, J.M., Kennan, A.L., and Porter, J.W. (1969) *J. Biol. Chem. 244,* 4510–4516.
37. Spence, J.T., Koudelka, A.P., and Tseng-Crank, J.C.L. (1985) *Biochem. J. 227,* 939–947.

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Effects of Cyclopropenoid Fatty Acids on Fungal Growth and Lipid Composition

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Cyclopropenoid fatty acids (CPE) isolated from *Sterculia foetida* oil by urea clathration and reverse phase high performance liquid chromatography (HPLC) were introduced into fungal cultures. Stearate levels in phospholipids and triacylglycerols from *Ustilago maydis* sporidia rose considerably in response to 30 μM CPE. In addition, CPE themselves were incorporated into glycerolipid fractions. Sterol composition was unaffected. Changes in lipid composition were accompanied by inhibition of dry weight accumulation and sporidial number. Treated sporidia showed irregular wall deposition and a branched morphology. Oleate alleviated CPE effects on growth and morphology. Hyphal extension by *Rhizoctonia solani* was inhibited somewhat by 30 μM sterculate, while *Fusarium oxysporum* showed no appreciable response. Although CPE appeared to inhibit fatty acid desaturation by *F. oxysporum*, gross increases in the proportion of stearate were limited to the triacylglycerol fraction during 30 μM treatments. The possibility that the CPE synthesized by plants serve as antifungal agents is discussed.

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Cyclopropenoid fatty acids (CPE), compounds synthesized both by plants allied with order Malvales and by certain gymnosperms (1–6), are well-known inhibitors of fatty acid desaturation in higher animals (6–8). Evidence that CPE also affect insect stearoyl-CoA desaturase (9,10) and interfere with the maturation and reproduction of some insect species (9–12) prompted suggestions that in addition to their traditional role as storage lipids, CPE also serve as protective agents (9,10).

Similarities between animal and fungal Δ^9 desaturase systems (13) led us to hypothesize that CPE also play an antifungal role in plants. Early work by Reiser et al. (14) indicated that *Saccharomyces cerevisiae* accumulates palmitate (16:0) at the expense of palmitoleate (16:1) when treated with CPE-containing *Sterculia foetida* oil. Recently, Moreton (15) reported that although tested *S. cerevisiae* strains did not respond to CPE these compounds increased the proportion of saturated fatty acids in several other yeasts. Here, we present evidence that CPE inhibit fatty acid desaturation in two fungi of interest to plant pathologists. We also report that low levels of CPE interfere with cytokinesis by sporidia of *Ustilago maydis*.

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Abbreviations: CPE, cyclopropenoid fatty acids; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

MATERIALS AND METHODS

Isolation of CPE methyl esters. Two CPE, sterculate (1-cyclopropene-1-octanoic acid, 2-octyl) and malvalate (1-cyclopropene-1-heptanoic acid, 2-octyl), were isolated from *Sterculia foetida* oil. The oil was transesterified with sodium methoxide, and the methyl esters sere subjected to urea clathration (16,17). Clathration mixtures containing 15 g urea, 4 ml methanol and 40 ml petroleum ether/g of methyl ester generated filtrates containing primarily sterculate, malvalate and linoleate (18:2). Individual CPE then were isolated by reverse phase high performance liquid chromatography (HPLC) on a Varian Model 5000 liquid chromatograph equipped with an 8 mm X 30 cm Varian MicroPak MCH-10 semipreparative column. Peaks were eluted with acetonitrile/water (82:18, v/v) at 6 ml/min and detected at 200 nm. Samples up to 15 μl gave separations similar to those obtained by Loveland et al. (18) using an analytical protocol. Although 16:0 and 18:1 overlap the sterculate peak under these conditions, removal of the bulk of these compounds by clathration and collection of the peak after the leading edge had reached half-height permitted sterculate preparations exceeding 97% purity by gas chromatography. Due to the relative stability of CPE methyl esters compared to free CPE, the purified methyl esters were used directly in fungal treatments. For the purposes of this study, it was hypothesized that metabolism of these methyl esters by fungi is analogous to metabolism of the natural glycerol esters.

Culture methods. Ustilago maydis (DC) Cda., ATCC 14826 sporidia were grown on a 200 rpm rotary shaker for periods up to 64 hr. Temperatures ranged from 26–30 C. The culture medium of Coursen and Sisler (19) was supplemented with 2.0 g KH_2PO_4 and 1.5 g K_2HPO_4 per liter (medium 72). Fatty acid methyl esters were added in 4 or 5 ml methanol per l; control flasks contained the corresponding amount of pure methanol.

Fusarium oxysporum Schlecht isolated by Kaufman and Blake (20) was maintained on potato dextrose agar. The liquid medium used for fatty acid analysis work contained 0.6 g KH_2PO_4, 0.2 g K_2HPO_4, 0.2 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 2.0 g sucrose and 1.0 g yeast extract per liter with an initial pH of 6.3 (21). Mycelium was transferred from a plate to liquid culture three days before an experiment and to fresh medium twice before inoculation of the test cultures. Methyl esters were added to the latter as above, and cultures were grown for 16 hr at 30 C on a 200 rpm rotary shaker. *Rhizoctonia solani* Kuhn AG 4, isolate SN-1 of A. Ogoshi, was grown on potato dextrose agar.

Morphological studies. Fluorescence microscopy was performed on a Leitz SM-LUX microscope with Epi-Fluorescence and a 100 W ultra-high pressure mercury lamp. Cell wall material was stained with 0.2% Calcofluor White (22) and observed using a UG-1

excitation filter, a TK400/K430 suppression filter and a K430/K460 barrier filter. Nuclei stained with mithramycin (23) were observed using a KP500 excitation filter, a TK510/K515 suppression filter and a K510/530 barrier filter.

Growth measurements. For dry weight measurements, 40–ml aliquots of liquid cultures were pelleted. Fungal material was washed twice with distilled water, transferred to tared pans, and dried at 80 C for 24 hr. For cell counts and viability studies, sporidia were diluted appropriately and vortexed briefly to minimize clumping. Any remaining clumps observed in the hemocytometer were registered as single branched cells. Viability tests were performed on medium 72 plates containing 20 g agar per liter. Colonies were counted after two to three days. Hyphal extension rates were determined on plates containing potato dextrose agar supplemented with either 30 μM methyl sterculate in methanol (4 ml/l medium) or methanol alone.

Lipid analysis. Ustilago maydis and *Fusarium oxysporum* harvested by centrifugation were washed twice with distilled water, flash frozen and lyophilized. Dry material was homogenized and extracted with chloroform/methanol (2:1, v/v) at room temperature for one hr with agitation. Extraction mixtures were supplemented with an internal standard containing tripentadecanoin, diheptadecanoyl phosphatidylcholine and methyl nonadecanoate.

Filtered extracts were separated into two fractions on BioSil A (BioRad, Richmond, CA) columns (24). Neutral lipids were eluted with chloroform followed by acetone. Phospholipids were flushed from the BioSil with methanol. Neutral lipids were further fractionated by thin layer chromatography (TLC) on aluminum–backed plates coated with 0.2 mm Silica Gel 60F-254 (EM Science, Cherry Hill, NJ) and developed with petroleum ether/diethyl ether (85:15) (25). Bands were visualized with Rhodamine 6G. For gas chromatography, glycerolipid fractions were transesterified with room temperature sodium methoxide (16). Free fatty acid fractions were methylated with boron trichloride/methanol (10% w/v) (Applied Science Lab, Deerfield, IL) at 60 C (26). Fatty acid methyl ester fractions from derivatization mixtures or from TLC plates were transferred to hexane, washed with water and dried over anhydrous sodium sulfate before gas chromatography.

Analyses were performed on a Varian 3700 Gas Chromatograph coupled with a Varian Vista 401 Chromatography Data System. Detection was carried out at 240 C by an Aerograph Flame Ionization Detector given 30 ml/min hydrogen and 300 ml/min air. With the exceptions of methyl oleate (18:1), methyl linoleate (18:2) and methyl linolenate (18:3), all methyl esters present in significant quantities could be separated on a 6 ft X 1/16 in ID glass column packed with 3% SP-2100 on 80/100 Supelcoport (Supelco Inc., Bellefonte, PA) (27). The injection temperature was 220 C, the column temperature 180 C and the helium flow 29 ml/min. The 18:1/18:2/18:3 ratio was determined at 210 C on a 60 m X 0.25 mm inside diameter fused silica capillary with a 1 micron film of SPB-1 (Supelco). The injection temperature was 220 C, the helium carrier flow 0.63 ml/min, and the split ratio 40/1. Helium make-up gas was supplied at 30 ml/min.

For sterol analysis, crude extracts were saponified under nitrogen and fractionated by alumina column chromatography (28). Sterols were quantitated by gas chromatography at 240 C on a 6 ft $\times$ 1/16 in glass column packed with 3% SE-30 on 100/120 Gas-Chrom Q (Applied Science). The injector temperature was 290 C, the detector temperature 300 C, and the helium flow 11 ml/min.

RESULTS

Lipid composition. CPE had two major effects on lipid compositions of *Ustilago maydis* and *Fusarium oxysporum*. First, the two fungi were able to incorporate CPE into both phospholipids (Table 1) and triacylglycerols (Table 2). CPE incorporation by *U. maydis* was more pronounced than incorporation by *F. oxysporum*. Traces of sterculate were detected in *U. maydis*

TABLE 1

Fatty Acid Composition of Phospholipids from *Ustilago maydis* and *Fusarium oxysporum*

Fatty acid species	Proportion of fatty acid species (%)[a]			
	Ustilago maydis		*Fusarium oxysporum*	
	Control	30 μM Sterculate	Control	30 μM Sterculate
14:0	0.8	0.8	0.2	0.3
16:0	16.8	11.9	14.8	15.7
16:1	5.1	0.8	0.6	0.3
18:0	2.4	22.2	1.0	3.4
18:1	15.8	7.1	10.1	8.0
18:2	59.0	35.6	54.3	52.2
18:3	n.d.	n.d.	18.9	18.6
Sterculate	n.d.	21.6	n.d.	1.4

[a]Values are averages from four cultures.
n.d., not detected.

TABLE 2

Fatty Acid Composition of Triacylglycerols from *Ustilago maydis* and *Fusarium oxysporum*

Fatty acid species	Proportion of fatty acid species (%)[a]			
	Ustilago maydis		*Fusarium oxysporum*	
	Control	30 μM Sterculate	Control	30 μM Sterculate
14:0	1.6	1.2	0.6	0.6
16:0	36.2	18.2	15.7	18.4
16:1	5.0	0.8	0.6	0.2
18:0	9.3	44.6	7.4	31.6
18:1	23.2	6.4	32.4	22.2
18:2	24.6	8.5	29.0	18.5
18:3	n.d.	n.d.	14.1	7.4
Sterculate	n.d.	20.4	n.d.	1.1

[a]Values are averages from four cultures.
n.d., not detected.

sporidia grown in 1 μM sterculate. After 30 μM treatments, CPE comprised more than 20% of glycerolipid fatty acids in this organism. Phospholipids and triacylglycerols from *F. oxysporum* given 30 μM sterculate contained less than 2% CPE. In all, roughly 10% of the CPE added to *F. oxysporum* cultures during 30 μM treatments was recovered from the fungal tissue. Per gram of lyophilized material, 0.2 mg sterculate was recovered in the original methyl ester form, another 0.2 mg from the triacylglycerols and 0.5 mg from the phospholipid fraction. *U. maydis*, on the other hand, accumulated 50% of the material added: 0.2 mg/g dry weight as methyl esters, 5.4 mg in the phospholipids and 5.1 mg in the triacylglycerols. No CPE were recovered in free fatty acids from either species, although the relative instability of free CPE (29–30) should be kept in mind. Malvalate accumulation by *U. maydis* was comparable to sterculate accumulation.

Acyl lipids from CPE-treated fungi also were more saturated than control lipids. *U. maydis* sporidia showed a clear increase in triacylglycerol 18:0 following 1 μM sterculate treatments, although phospholipid fatty acids were relatively unaffected. When cultures were supplemented with 30 μM sterculate, the proportion of 18:0 in triacylglycerols rose from 9 to 44% in *U. maydis* and from 7 to 32% in *F. oxysporum* (Table 2). The proportion and quantity of 18:0 in *U. maydis* phospholipids also increased substantially under these conditions, whereas *F. oxysporum* phospholipids were relatively unaffected by 30 μM sterculate (Table 1).

Despite the large shifts in *U. maydis* fatty acid composition induced by 30 μM sterculate or malvalate, sterol profiles of treated sporidia were indistinguishable from those of controls. Ergosterol, the primary sterol in all samples examined, remained at levels around 2.4 mg per gram dry wt.

Growth and morphology. On plates containing 30 μM sterculate, *Rhizoctonia solani* AG4 hyphae grew at about three-fourths of their rate on control plates. Hyphal extension by *F. oxysporum* was unaffected under the same conditions. Dry wt of *F. oxysporum* in liquid cultures also showed no consistent inhibition by 30 μM sterculate. Reduced dry weight accumulation by *U. maydis* cultures was evident at CPE concentrations as low as 10 μM and approached a maximum inhibition at concentrations greater than 30 μM (Fig. 1). There was no indication that *U. maydis* sporidia differed in their response to sterculate and malvalate.

Progress in dry weight accumulation and sporidial number during batch culture of *U. maydis* is illustrated in Figure 2. Whereas dry weights in cultures treated with 30 μM sterculate diverged from those in control cultures only after the latter had reached the late log phase, the number of sporidia in treated cultures was severely depressed within 12 hr and remained virtually unchanged while control cultures passed through log phase and into stationary phase. That cultures eventually recovered from the treatment is evident by either measurement and probably reflects depletion of CPE from the medium.

Morphologically, almost all control sporidia were cigar-shaped and divided longitudinally (Fig. 3A); they retained these characteristics in the presence of

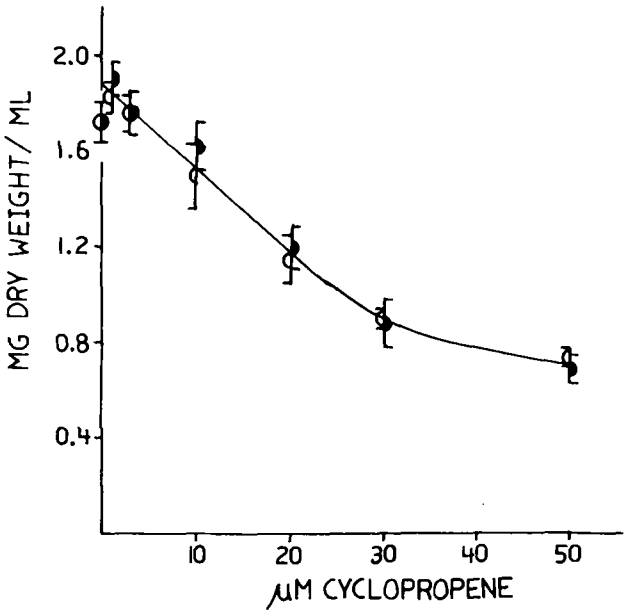

FIG. 1. Effects of CPE methyl ester concentration on growth of *Ustilago maydis* sporidia (mg dry wt/ml culture medium). Cells were harvested after 20 hr growth, washed twice with distilled water and dried at 80 C. ∮ = sterculate; ∮ = malvalate. Bars represent standard errors of six flasks.

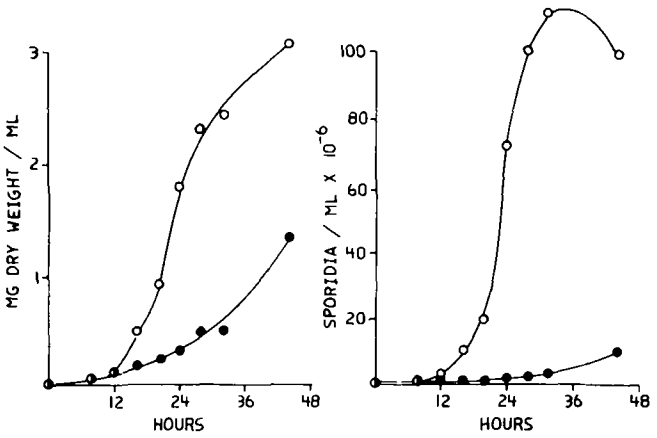

FIG. 2. Growth of *U. maydis* sporidia in the presence of 30 μM methyl sterculate. A, Dry wt accumulation over time (mg/ml). B, Changes in number of sporidia over time (sporidia/ml). Sporidia were grown on a 200 rpm rotary shaker at 30 C. Points represent average values for four flasks from two experiments. ○, control; ●, 30 μM sterculate treatment.

methyl 16:0, methyl 18:1 or methyl 18:2. In contrast, populations treated with methyl CPE were heterogeneous. Some cells were indistinguishable from control cells. Other sporidia became excessively elongate, while many became branched and tended to clump. Among branched sporidia, simple Y-shapes predominated at earlier times. Increasingly, bizarre forms and clumping became common as the treatment progressed. Figures 3B-D illustrate sporidia showing swollen areas and varying degrees of branching.

The morphological symptoms discussed appear linked to a failure in cytokinesis. Mithramycin staining revealed multiple nuclei in both branched and ab-

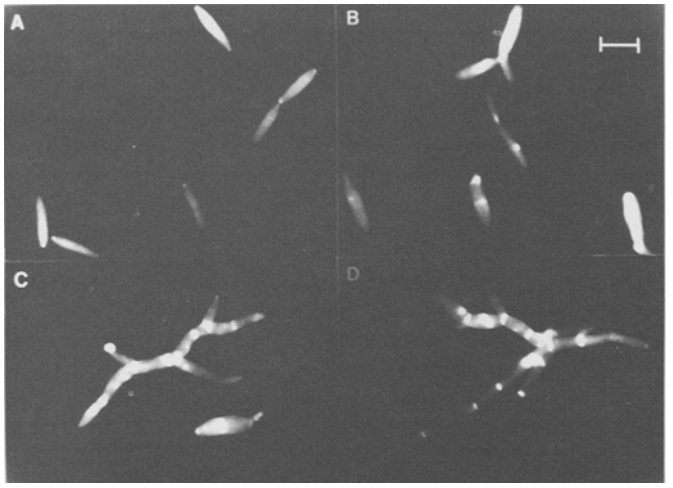

FIG. 3. Calcofluor staining of *U. maydis* sporidia. A, control sporidia; B-D, treated sporidia; bar, 10 μm.

normally long sporidia. Ordinary cigar-shaped cells had one or in cells approaching division two nuclei. Calcofluor staining of cell walls showed that the CPE-affected cells were multicellular (Figs. 3B-D). Septum formation did occur but the daughter cells failed to pinch off. Irregular patches of staining along the walls of treated cells may indicate abnormalities in the deposition of wall material.

The maximum number of *U. maydis* colony forming units in the presence of CPE would be limited both by the reduced number of sporidia available and by their viability. Approximately 30% of sporidia in cultures in which 22% of individuals were branched failed to produce colonies.

Relief of CPE effects by oleate. The effects of sterculate on sporidia could be mitigated by 18:1 (Table 3). Under conditions in which 30 μM sterculate reduced dry weight production to 47% of the control level, cultures given 30 μM sterculate plus 30 μM 18:1 showed normal dry wt accumulation. The number of sporidia and the proportion of branched sporidia in cultures treated with the fatty acid mixture were intermediate between values for controls and for cultures given only sterculate.

DISCUSSION

As discussed earlier, CPE inhibit Δ^9 fatty acyl desaturase in a number of organisms and might be expected by analogy to affect desaturation in fungi. In vertebrate and insect systems, various secondary disorders also are associated with CPE ingestion (6,9–12). This research was undertaken to determine whether CPE shift fatty acid profiles in fungi and, if so, whether they are detrimental to the organisms. It was hypothesized that CPE play a role as protective agents in plants.

The elevated 18:0 levels expected if CPE inhibit stearoyl-CoA desaturase were observed both in *Ustilago maydis*, the basidiomycete responsible for corn smut, and in *Fusarium oxysporum*, a filamentous imperfect fungus. In both organisms, triacylglycerol fatty acid composition responded to CPE more readily than did phospholipid composition, although both fractions showed a sizable response when *U. maydis* sporidia were treated with 30 μM CPE. Increased saturation of bulk lipid also has been reported to follow CPE treatment of some basidiomycetous and ascomycetous yeasts (14,15). A second effect of CPE on lipid composition, incorporation of the compounds into triacylglycerols and phospholipids, has not been reported previously for fungal systems (14,15). CPE are found in acyl lipids from treated vertebrates (31,32) and cultured hepatoma cells (33) but accumulate preferentially in the triacylglycerol fraction (32,33).

Dry weight accumulation by *U. maydis* cultures was inhibited by CPE levels as low as 10 μM. In animal systems, both desaturation in vitro and secondary effects in vivo are less responsive to malvalate than to sterculate (7,31). However, in this study there was no indication that *U. maydis* differentiated between the two. Plots of dry weight accumulation vs methyl sterculate and methyl malvalate concentrations (Fig. 1) were indistinguishable at a 5% confidence level. Increases in 18:0 and CPE incorporation also were similar for the two compounds.

The morphological responses of *U. maydis* sporidia to CPE were very similar to those reported for sporidia deficient in ergosterol. Sporidial branching is characteristic both of cultures exposed to fungicides inhibiting ergosterol biosynthesis (34) and of a mutant blocked at the C-14 demethylation step (35). In the same vein, Calcofluor staining and electron microscopy reveal irregular wall deposition in fungicide-treated *Ustilago* (36,37) and other fungi (38–40). Inhibition of ergosterol synthesis by CPE, therefore, might have been expected, particularly given the decreased levels of cytochrome P450 in trout fed the compounds (41). Nevertheless, sterol profiles of sporidia treated with 30 μM CPE mirrored those of control cells.

At the same time, it seems unlikely that these paral-

TABLE 3

Effects of Oleate on CPE-Induced Symptoms in *U. maydis* Sporidia

Treatment	Dry weight (mg/ml)	Sporidia/ml	% Branched sporidia
Control	0.32 ± 0.09	$(11.9 \pm 3.2) \times 10^6$	3.4 ± 1.4
30 μM Sterculate	0.15 ± 0.02	$(1.8 \pm 0.8) \times 10^6$	17.9 ± 3.0
30 μM 18:1	0.34 ± 0.08	$(13.3 \pm 3.9) \times 10^6$	4.2 ± 0.5
30 μM Sterculate + 30 μM 18:1	0.31 ± 0.03	$(9.0 \pm 2.0) \times 10^6$	7.6 ± 1.1

lels are coincidental. Discussions of ergosterol deficiency symptoms often note that wall deposition requires membrane-bound enzymes and suggest a link between altered membrane composition and changes in fungal morphology (34,37,40,42). A similar argument would hold for CPE-treated *U. maydis*, in which comparable irregularities are accompanied by severely altered phospholipid fatty acids. The contention that changes in fatty acid composition ultimately are responsible for CPE-induced changes in morphology is supported by the observation that 18:1, the product of the desaturation reaction blocked by CPE, suppresses development of the symptoms.

Whatever the specific mechanisms by which CPE affect *U. maydis* growth and morphology, the observation that they do so is consistent with the hypothesis that CPE serve as antifungal agents. Not all fungi are equally sensitive: when *Rhizoctonia solani* and *Fusarium oxysporum* were grown under identical conditions, hyphal extension by *R. solani* was somewhat inhibited by 30 μM CPE, while *F. oxysporum* appeared to grow normally. The resistance of *F. oxysporum* may be linked to an ability to degrade or exclude the compounds, since only about 10% of the CPE added to liquid cultures of the fungus was recovered. Also of interest is the finding that in contrast to the situation in the sensitive *U. maydis*, *F. oxysporum* phospholipids were relatively stable in the presence of 30 μM CPE. Variations in fungal susceptibility to CPE also have been noted by Moreton (15), who found that while most yeasts tested grew normally except at very high CPE concentrations some strains of *Lipomyces* were unable to grow in their presence.

Further work is needed to determine the range of fungi reacting to CPE and the levels of these compounds available to the organisms in vivo. We find that CPE levels in roots of several Malvaceous species exceed those used in this study (43). The observation that 18:1 will alleviate symptoms induced in *U. maydis* by CPE suggests that the fatty acid background in which CPE occur also will be significant. The response of fungi to natural CPE-containing mixtures is likewise of interest given that at least some CPE-producing plants also synthesize low levels of epoxy fatty acids (3), compounds that are fungistatic against *Pyricularia oryzae* (44).

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REFERENCES

1. Gaydou, E.M., and Ramanoelina, A.R.P. (1984) *Fette Seifen Anstrichm. 86*, 82–87.
2. Gaydou, E.M., and Ramanoelina, A.R.P. (1983) *Phytochemistry 22*, 1725–1728.
3. Bohannon, M.B., and Kleiman, R. (1978) *Lipids 13*, 270–273.
4. Vickery, J.R., Whitfield, F.B., Ford, G.L., and Kennett, B.H. (1984) *J. Am. Oil Chem. Soc. 61*, 573–575.
5. Vickery, J.R. (1980) *J. Am. Oil Chem. Soc. 57*, 87–91.
6. Christie, W.W. (1970) in *Topics in Lipid Chemistry* (Gunstone, F.D., ed.) Vol. 1, pp. 1–49, Logos, London.
7. Allen, E., Johnson, A.R., Fogerty, A.C., Pearson, J.A., and Shenstone, F.S. (1967) *Lipids 2*, 419–423.
8. Jeffcoat, R., and Pollard, M.R. (1977) *Lipids 12*, 480–485.
9. Binder, R.G., and Chan, B.G. (1982) *Ent. Exp. Appl. 31*, 291–295.
10. Thompson, S.N., and Barlow, J.S. (1973) *Ann. Entomol. Soc. Am. 66*, 797–800.
11. Beroza, M., and LaBrecque, G.C. (1967) *J. Econ. Entomol. 60*, 196–199.
12. Lang, J.T., and Treece, R.E. (1971) *J. Econ. Entomol. 64*, 455–457.
13. Weete, J.D. (1980) in *Lipid Biochemistry of Fungi and Other Organisms*, Plenum Press, New York, NY, 111-117.
14. Reiser, R., Parekh, C.K., and Meinke, W.W. (1963) in *Biochemical Problems of Lipids* (Frazer, A.C., ed.) pp. 251–256, Elsevier, Amsterdam, The Netherlands.
15. Moreton, R.S. (1985) Appl. Microbiol. Biotechnol. 22, 41–45.
16. Christie, W.W. (1982) in *Lipid Analysis*, 2nd ed., Pergamon Press, New York, NY, 53, 54,90-92.
17. Kircher, H.W. (1964) *J. Am. Oil Chem. Soc. 41*, 4–8.
18. Loveland, P.M., Pawlowski, N.E., Libbey, L.M., Bailey, G.S., and Nixon, J.E. (1983) *J. Am. Oil Chem. Soc. 60*, 1786–1788.
19. Coursen, B.W., and Sisler, H.D. (1960) *Am. J. Bot. 47*, 541–549.
20. Kaufman, D.D., and Blake, J. (1973) *Soil Biol. Biochem 5*, 297–308.
21. Reichel, H.D. (1986) M.S. Thesis, Inducers, Substrates and Inhibitors of a Propanil-Degrading Amidase of *Fusarium oxysporum*, University of Maryland, College Park, 4.
22. Bottino, P.J. (1981) in *Methods in Plant Tissue Culture*, Botany Dept., University of Maryland, College Park, 67.
23. Slater, M.L. (1976) *J. Bacteriol. 126*, 1339–1341.
24. Rouser, G., Kritchevsky, G., Simon, G., and Nelson, G.J. (1967) *Lipids 2*, 37–40.
25. Harrington, K.J., and D'Arcy-Evans, C. (1985) *J. Am. Oil Chem. Soc. 62*, 1009–1013.
26. Klopfenstein, W.E. (1971) *J. Lipid Res. 12*, 773–776.
27. Fisher, G.S., and Schuller, W.H. (1981) *J. Am. Oil Chem. Soc. 58*, 943–946.
28. Chiu, P.-L., Patterson, G.W., and Fenner, G.P. (1985) *Phytochemistry 24*, 263–266.
29. Nunn, J.R. (1952) *J. Chem. Soc.*, 313–318.
30. White, J.L., Sr., Zarins, A., and Feuge, R.O. (1976) *J. Am. Oil Chem. Soc. 54*, 335–338.
31. Pawlowski, N.E., Hendricks, J.D., Bailey, M.L., Nixon, J.E., and Bailey, G.S. (1985) *J. Agric. Food Chem. 33*, 767–770.
32. Roehm, J.N., Lee, D.J., Sinnhuber, R.O., and Polityka, S.D. (1971) *Lipids 6*, 426–430.
33. Zoeller, R.A., and Wood, R. (1984) *Lipids 19*, 529–538.
34. Kato, T. (1986) in *Chemistry of Plant Protection. 1. Sterol Biosynthesis Inhibitors and Anti-Feeding Compounds*, pp. 1–24, Springer-Verlag, Berlin.
35. Walsh, R.C., and Sisler, H.D. (1982) *Pestic. Biochem. Physiol. 18*, 122–131.
36. Hippe, S., Buchenauer, H., and Grossman, F. (1980) *Z. Pflanzenkr. Pflanzenschutz 87*, 423–426.
37. Kerkenaar, A., and Barug, D. (1984) *Pestic. Sci. 15*, 199–205.
38. Barug, D., Samson, R.A., and Kerkenaar, A. (1983) *Arzneim.-Forschung/Drug Res. 33*, 528–537.
39. Pring, R.J. (1984) *Pestic. Biochem. Physiol. 21*, 127–137.
40. Richmond, D.V. (1984) *Pestic. Biochem. Physiol. 21*, 74–83.
41. Eisele, T.A., Nixon, J.E., Pawlowski, N.E., and Sinnhuber, R.O. (1978) *J. Environ. Pathol. Toxicol. 1*, 773–778.
42. Buchenauer, H. (1977) *Pestic. Biochem. Physiol. 7*, 309–320.
43. Schmid, K.M. (1987) Ph.D. Thesis, Effects of Cyclopropenoid Fatty Acids on Fungal Growth and Lipid Composition, University of Maryland, College Park, 16-26, 66, 67.
44. Kato, T., Yamaguchi, Y., Abe, N., Uyehara, T., Namai, T., Kodama, M., and Shiobara, Y. (1985) *Tetrahedron Lett. 26*, 2357–3260.

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METHODS

Quantitation of 1,2-Diacylglycerol in Rat Heart by Iatroscan TLC/FID

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1,2-Diacylglycerol, which has been recognized as one of the intracellular second messengers, was measured quantitatively in the lipid extract from rat hearts using the thin layer chromatography and flame ionization detection (TLC/FID) method. Cholesterol acetate was added to the tissue as an internal standard, and the crude lipids from the tissue were purified with silicic acid column to eliminate phospholipids. Development of Chromarods was carried out using two solvent systems and a three-step development technique. The relationship of the peak area ratio detected by flame ionization detector to weight ratio was linear compared with cholesteryl acetate. The 1,2-diacylglycerol content in the rat heart in the unstimulated condition was 72.5 ± 15.3 ng/mg wet wt (mean ± SD).

Lipids 23, 253–255 (1988).

It is accepted widely that hormones and neurotransmitters that elicit intracellular mobilization of Ca^{++} cause phosphoinositide hydrolysis by phospholipase C. The products of this reaction, inositol triphosphate and 1,2-diacylglycerol (DG), both play an important role in the signal transduction process (1,2). An increase in 1,2-DG content in the tissue is thought to have multiple metabolic roles. 1,2-DG produces the activation of protein kinase C and releases fatty acids including arachidonate. Accordingly, 1,2-DG has been recognized as one of the second messengers in the transmembranous cellular signal transduction process. Despite recent interest in the role of 1,2-DG, very few direct measurements have been made on the extent and duration of the 1,2-DG accumulation in response to hormones because of the small amount in the tissue. Therefore, it is difficult to estimate the exact amount of 1,2-DG. In particular, the function and role of 1,2-DG accumulation in the myocardium is unknown. Although lipids labeled with radioactive fatty acids, followed by separation of 1,2-DG by thin layer chromatography (TLC), have been used often (3), this method does not provide a quantitative estimate of 1,2-DG content. For quantitative measurement for tissue 1,2-DG, TLC (4), gas liquid chromatography mass spectrometry (5), and high performance liquid chromatography (HPLC) have been used (6). We have employed a modification of the TLC/FID (flame ionization detection) method for a reliable quantitative estimate of tissue 1,2-DG.

MATERIALS AND METHODS

Materials. 1,2,3-Tri-*cis*-9′-octadecenoyl-*rac*-glycerol (triolein), *cis*-9′-octadecenoic acid (oleic acid), cholesterol, 1,3-di-*cis*-9′-octadecenoyl-*rac*-glycerol (1,3-diolein), 1,2-di-*cis*-9′-octadecenoyl-*rac*-glycerol (1,2-diolein) and 1-*cis*-9′-octadecenoyl-*rac*-glycerol (monoolein) were purchased from the Sigma Chemical Co. (St. Louis, MO). Cholesteryl acetate and butylated hydroxytoluene were obtained from the Wako Chemical Co. (Osaka, Japan). All other chemicals were of analytical reagent grade. Adult male Wistar rats weighing 200–300 g were deprived of food for 12 hr overnight but allowed free access to water. Following decapitation, the heart rapidly was excised and washed with normal saline. A 60–80 mg tissue sample from the left ventricle near the apex was removed and frozen rapidly in liquid N_2.

Instrumentation. Sample analyses were carried out with the Iatroscan TH-10 TLC/FID analyzer (Iatron Inc., Tokyo, Japan) under the following conditions: flow-rate of hydrogen, 160 ml/min; flow-rate of air, 2000 ml/min; and a scan speed, 2.39 sec/cm. Silicagel, 75-μm precoated thin-layer rods (Chromarods-SII, Iatron Inc., Tokyo, Japan) were in sets of 10. Peak areas were calculated using a potentiometric recorder (Chromatocorder 11, System Instrument, Tokyo, Japan) connected to the analog output of the Iatroscan TH-10.

Neutral lipid extraction. Lipids were extracted by the method of Folch et al. (7) with some modification. The sample was placed in 5.5 ml ice-cold chloroform/methanol mixture (2:1, v/v) containing 0.01% butylated hydroxytoluene as an antioxidant and cholesteryl acetate (0.1 mg per tube) as an internal standard, and homogenized in a motor-driven Potter-Elvehjem homogenizer kept in ice for 20 sec as soon as the tissue began to soften. The homogenate was chilled on ice for 30 min and filtered through Toyo filter paper. The filtrate was evaporated to dryness under a stream of N_2 at 30 C. Dried lipids were resuspended in 500 μl of chloroform and applied to 0.5-ml silicic acid (minus 325 mesh from Bio-Rad, Richmond, CA) column (20 mm x 5.5 mm), equilibrated with chloroform. Neutral lipids and free fatty acids were eluted thoroughly with 6 ml chloroform, concentrated under a stream of N_2 at 30 C, and dissolved in 20 μl chloroform.

Chromarod development and scanning. The Chromarods were activated by passing through the Iatroscan TH-10 analyzer before use. Two μl of the lipid extracts was applied carefully to the rods with a 10 μl Hamilton syringe. The set of rods was equilibrated before each development for five min over the developing solvent. The first development was carried out in a solvent system of 1,2-dichloroethane/chloroform/acetic acid (46:8:0.05, v/v/v) until the solvent front had migrated ca. 9 cm. The rods were air-dried at room temperature for 15 min, then were subjected to the second development in the same way as the first one. After the rods were dried as above for 15 min, the last development was carried out in a solvent system of *n*-hexane/diethyl ether/acetic acid (98:2:1, v/v/v) in the same direction until the solvent front had mi-

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Abbreviations: DG, diacylglycerol; FID, flame ionization detection; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

grated ca. 11 cm. Chromarods then were dried at 50 C and scanned in the Iatroscan TH-10. Each sample was analyzed with four Chromarods, and the results were averaged.

RESULTS AND DISCUSSION

The separation pattern of the standard mixture under the above mentioned condition is shown in Figure 1. The relative mobilities (R_f values) of butylated hydroxytoluene, cholesteryl acetate, triolein, oleic acid, cholesterol, 1,3-diolein and 1,2-diolein (ca. 0.4:2:1:1:1:1:1; by weight) were 0.90, 0.75, 0.63, 0.50, 0.35, 0.24, and 0.16, respectively. However, the R_f value of each authentic lipid upon separate developing was a little different from that in the standard mixture solution. Monoolein migrated a little (R_f=0.03, data not shown). These standard mixtures gave a very good separation of neural lipids. Figure 2A shows the peaks of 1,2-diolein and cholesteryl acetate (1:15, w/w) standard mixture solution. After the entire extraction procedure utilized here for rat hearts, the ratio of 1,2-diolein/cholesteryl acetate was not changed compared with that before the extraction as shown in Figure 2B. The recovery of 1,2-diolein by the extraction involving the silicic acid column procedure compared with cholesteryl acetate (i.e. the change in the ratio of the two) was 99.5±9.4% (SD, n=4), indicating that the intraassay coefficient of variation was 9.5% in the extraction and assay according to this method. The cholesteryl acetate and 1,2-DG peaks also were separated well in the extract from rat hearts containing cholesteryl acetate as an internal standard although triglycerides and free fatty acids were not clearly separated (Fig. 3A), and 1,2-diolein (1/15 of cholesteryl acetate, w/w) added to the tissue before the extraction migrated on the same peak of 1,2-DG in the myocardium, confirming that peak b in Figure 3A corresponds to the 1,2-DG peak (Fig. 3B). The standard curve for weight ratio and peak area ratio of 1,2-diolein to cholesteryl acetate is illustrated in Figure 4 and conform to the linearity throughout the ratio range tested. On the basis of the improved TLC/FID method, 1,2-DG content in rat hearts was 72.5±15.3 ng/mg wet wt (mean ± SD, n=8), whereas cholesterol content was 1.77±0.17 μg/mg wet wt (n=8).

Recently, it has been suggested that 1,2-DG is formed from phosphatidylcholine by agonists that stimulate protein kinase C as well as from phospho-

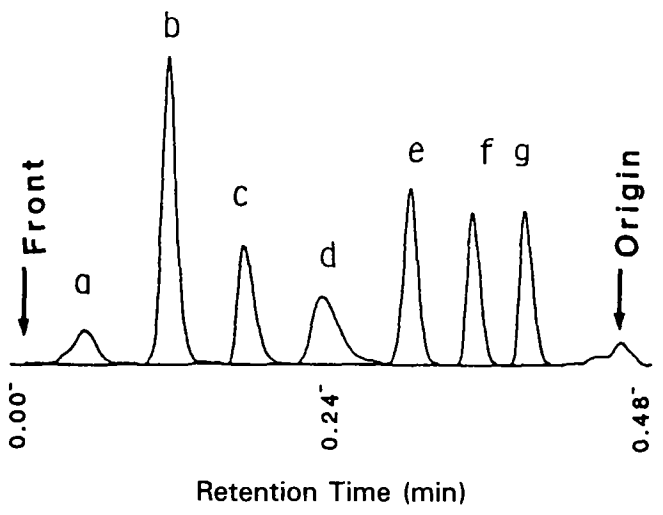

FIG. 1. TLC/FID analysis of the standard lipids. The peaks are as follows: a, butylated hydroxytoluene; b, cholesteryl acetate; c, triolein; d, oleic acid; e, cholesterol; f, 1,3-diolein; g, 1,2-diolein. First and second developments in 1,2-dichloroethane/chloroform/acetic acid (46:8:0.05, v/v/v) to 9 cm; third development in *n*-hexane/diethyl ether/acetic acid (98:2:1, v/v/v) to 11 cm.

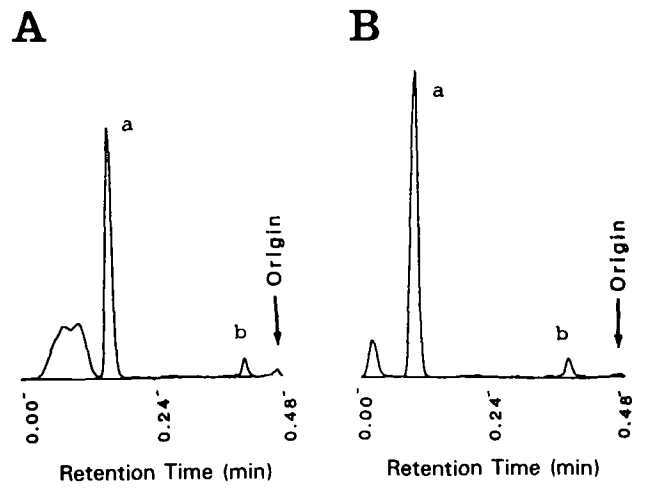

FIG. 2. TLC/FID analysis of 1,2-diolein (b) and cholesteryl acetate (a) standard mixture (1:15, w/w) with (B) and without (A) silicic acid column procedure, indicating that silicic acid column chromatography will not affect adversely the quantitative recovery of these compounds.

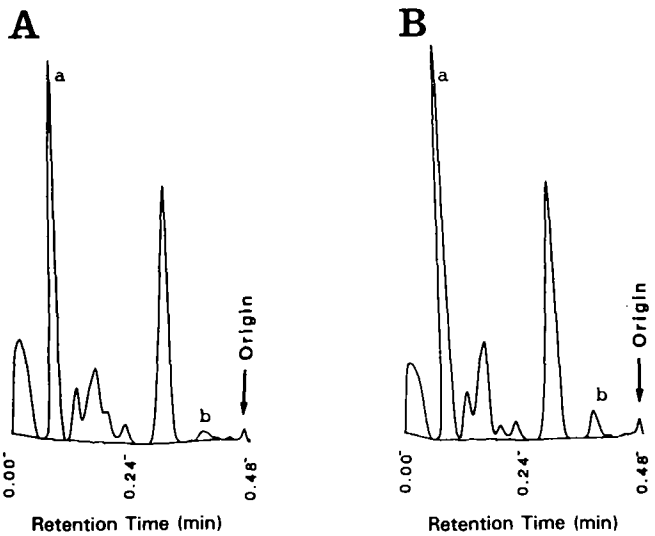

FIG. 3. TLC/FID separation of the tissue sample from rat heart (A) and the 1,2-diolein added tissue sample (B) (1:15 ratio of the added 1,2-diolein amount *[b]* to cholesteryl acetate as an internal standard *[a]*).

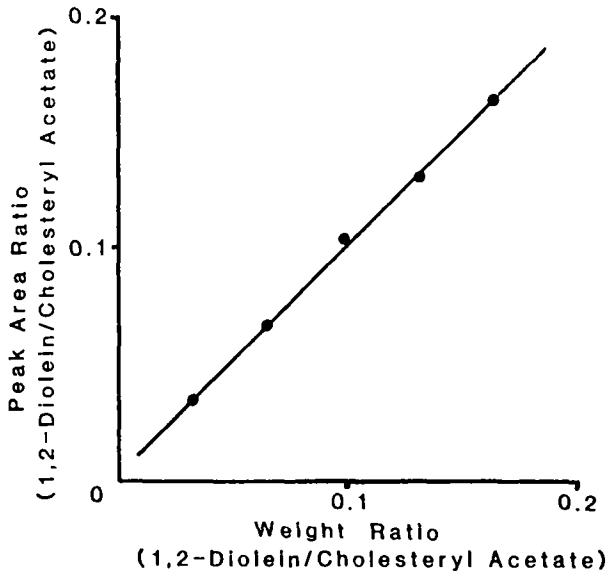

FIG. 4. The relationship between weight ratio and peak area ratio of 1,2-diolein to cholesteryl acetate by the FID response.

inositide hydrolysis (8). 1,2-DG is also a central intermediary metabolite in phospholipid and triglyceride synthesis. Despite recent interest in the role of phosphoinositide hydrolysis and 1,2-DG accumulation, few direct measurements of 1,2-DG have been made in tissue. Labeling of lipids with radioactive fatty acids may lead to mistaken conclusions about changes in 1,2-DG content when specific activity of the precursors and the fatty acid composition of 1,2-DG are unknown. The HPLC method for the estimation of 1,2-DG content has been developed recently (6,9), but TLC methods that have been used widely for lipid separation reveal a 1,2-DG spot isolated from other lipids, and they are very simple and fast. The difficulty in using the TLC method is quantitative estimation of 1,2-DG, because of the need for scraping plates and then re-extracting lipids. Therefore, accurate evaluation of 1,2-DG content is likely to be compromised.

This study established the method using TLC/FID to evaluate the amount of 1,2-DG. The phospholipid composition has been examined in the myocardium using TLC/FID (10,11). Christie and Hunter (12) have shown a separation of neutral lipids by means of the Iatroscan TH-10 analyzer. We chose cholesteryl acetate as an internal standard to enable quantitative estimation of 1,2-DG and cholesterol content. A three-step development of the neutral lipids on Chromarods in the same direction gave a good separation. The neutral lipids were separated by a silicic acid column because, in the presence of phospholipids, the resolution of 1,2-DG is not clear. Based on the analysis of the improved TLC/FID methods presented here, the 1,2-DG content in the myocardium is one-third and one-half of those previously reported for gerbil brain with TLC (13) and for isolated hepatocytes with HPLC (9), respectively. However, it almost is compatible with those from gerbil brain using HPLC (6) and from rat hearts using TLC (14). Both studies utilized a quick freezing and careful extraction method. This discrepancy of the control value of 1,2-DG content in tissues not only is due to differences among species and organs but may be due to the technique used to extract 1,2-DG.

In summary, we demonstrated a method for determining 1,2-DG content in myocardial lipids using a TLC/FID technique.

REFERENCES

1. Berridge, M.J. (1984) *Biochem. J. 220*, 345–360.
2. Nishizuka, Y. (1984) *Science 225*, 1365–1370.
3. Banschbach, M.W., Geison, R.L., and O'Brien, J.F. (1974) *Anal. Biochem. 59*, 617–627.
4. Freeman, C.P., and West, D. (1966) *J. Lipid Res. 7*, 324–327.
5. Satouchi, K., and Saito, K. (1976) *Biomed. Mass Spectrom. 3*, 122–126.
6. Abe, K., and Kogure, K. (1986) *J. Neurochem. 47*, 577–582.
7. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497–509.
8. Besterman, J.M., Duronio, V., and Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. USA 83*, 6785–6789.
9. Bocckino, S.B., Blackmore, P.F., and Exton, J.H. (1985) *J. Biol. Chem. 260*, 14201–14207.
10. Kramer, J.K.G. (1980) *Lipids 15*, 651–660.
11. Okumura, K., Ogawa, K., and Satake, T. (1983) *J. Cardiovasc. Pharmacol. 5*, 983–988.
12. Christie, W.W., and Hunter, M.L. (1979) *J. Chromatogr. 171*, 517–518.
13. Porcellati, G., DeMeddio, G.E., Fini, C., Floridi, A., Goracci, G., and Horrocks, L.A. (1978) *Proc. Eur. Soc. Neurochem. 1*, 285–302.
14. Kramer, J.K.G., and Hulan, H.W. (1978) *J. Lipid Res. 19*, 103–106.

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COMMUNICATIONS

Long-chain Alkanediols: Biological Markers for Cyanobacterial Contributions to Sediments

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One significant family of sedimentary lipids of widespread occurrence are series of C_{28}-C_{32} alkanediols and hydroxyketones. The recognition of the same series of alkanediols as major lipid components of a field population of the cyanobacterium *Aphanizomenon flos-aquae* leads us to propose that these lipids are markers for cyanobacterial inputs to sediments. The frequency of occurrence of the alkanediols in sedimentary environments supports the recent finding that cyanobacteria are major contributors to the aquatic biomass.

Lipids 23, 256-258 (1988).

An increasing number of the lipid components recognized in sediments have become established markers of contributions from specific organisms (1,2). For example, sedimentary 4-methylsterols (3–5) and 4-methylsterones (5) are derived from dinoflagellates, various acyclic isoprenoids are indicators of methanogenic (6) or other archaebacteria (7), long-chain alkenones originate from prymnesiophyte algae (8,9) and *n*-alkane distributions with an odd/even predominance in the C_{27}–C_{33} range denote contributions from higher plants (10).

Long-chain C_{28}-C_{32} alkane-1,15-diols together with a series of related 15-oxyalkan-1-ols first were recognized in Unit 1 sapropellic sediments from the Black Sea (11). Their structures were deduced from the mass spectral characteristics of their trimethylsilyl derivatives by analogy with synthetic, shorter chain standards (i.e. octadecane-1,12-diol and 12-oxyoctadecan-1-ol) (11). Similar series of alkanediols now have been found, often as major components, in a wide range of marine sediments (Table 1) and also have been observed in both recent (17,18, N. Robinson, G. Eglinton and P.A. Cranwell, unpublished data) and ancient (17) lacustrine sediments. Typically, they range from C_{28} to C_{32} with the mid-chain hydroxyl group in a number of positions (12) but dominantly at C-13 and C-15 for the C_{28} and C_{29}, and C_{30}-C_{32} components, respectively. The biological origins of these significant compounds and their related oxyalkanols have been the subject of much speculation and interest (11–13,19,20), which is resolved by this report of their discovery in a field population of the cyanobacterium *Aphanizomenon flos-aquae*.

TABLE 1

Occurrence of Alkanediols in the Extracts of Marine Sediments[a]

Location	Age	C No. Range[b]	Reference
Black Sea	Holocene	28-32	11
Mediterranean	Holocene	28-32	12
Mediterranean	Holocene	26-32	15
Baltic Sea	Holocene	28-32	Fig. 1
NW African Shelf	Holocene	28-32	Morris, unpublished
Arabian Slope	Holocene	28-32	Morris, unpublished
Guinea Basin	Holocene	28-32	Morris, unpublished
Gulf of California	Quaternary	28-32	Brassell, unpublished
Cariaco Trench	Quaternary	30	Brassell, unpublished
Mediterranean	Pleistocene	28-32	15
Middle America Trench	Pleistocene	30	Brassell, unpublished
Orca Basin	Pleistocene	30	Brassell, unpublished
Walvis Ridge	Pleistocene	28-34	Brassell, unpublished
Mediterranean	Pleistocene/ Pliocene	28-32	16
Japan Trench	Pleistocene/ Pliocene/ Miocene	26-32	Brassell, unpublished

[a]All of the sediments cited also contain series of hydroxyketones.
[b]Triaconta-1,15-diol (C_{30}) is the major component in all of the sediments, except the Arabian Slope where triaconta-1,14-diol dominates.

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Abbreviations: GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; TMS, tetramethylsilane.

EXPERIMENTAL

Samples were collected during a research cruise of RV Aranda in the Baltic Sea (59°50.99'N, 24°49.88'E) on July 25, 1984, while a virtually unispecies bloom of *Aphanizomenon flos-aquae* (M. Huttunen, personal communication) was in progress. Water was pumped from 3 m depth and passed through a continuous-flow spinning cup centrifuge (21,22) at a flow rate of 5 liters/hr^{-1}. Under these conditions, the effluent contains no particles > 1 μm (22). The wet cells were removed from the cup, an aliquot was taken for phytoplankton counting and identification and the remainder was placed directly in $CHCl_3$/MeOH (2:1, v/v) at - 30 C under N_2. Lipids were extracted (23), saponified and separated into acidic and neutral lipid fractions (24). Both fractions were analyzed by gas chromatography (GC), after methylation (BF_3/MeOH) and silylation (BSTFA), respectively, using a Carlo Erba 4160 instrument equipped with an on-column injector and fitted with a SE-30 flexible silica capillary column (25 m × 0.3 mm id), temperature programmed from 40–100 C at 8 C min^{-1} and then 100–280 C at 4 C min^{-1}. The detector was held at 300 C and the injector at 40 C. Lipid identification was made by gas chromatography-mass spectrometry (GC-MS), employing a Carlo-Erba Mega chromatograph equipped with an on-column injector and fitted with an OV-1 flexible silica column (22 m), temperature programmed from 50–140 at 6 C min^{-1}, then 140–300 at 4 C min^{-1}, and interfaced directly to a Finnigan 4000 quadrupole mass spectrometer scanned from m/z 50-600 at one sec intervals. Data acquisition and processing utilized an INCOS 2300 data system.

RESULTS AND DISCUSSION

Examination of the extractable lipids revealed an assemblage typical of phytoplankton. The principal constituents were phytol, carboxylic acids, sterols, alkanols and hydrocarbons (Table 2) together with a significant proportion of C_{28}-C_{32} alkanediols. The alkanediols were characterized from the mass spectra of their tetramethysilane (TMS) ethers that permitted elucidation of the position of the mid-chain hydroxyl group and the presence of double bonds from the m/z values of key fragment ions (11,12). Such mass spectral features enabled the recognition of the various isomers present (e.g. 1,13-,1,14- and 1,15-triacontanediols), despite their GC coelution. The range of alkanediols in the cyanobacterium population (Fig. 1) was similar to that observed previously in sediments from the Mediterranean (12) and elsewhere (Table 1); the dominant C_{28} and C_{29} components were 1,13-diols, whereas the C_{30}-C_{32} components were principally 1,15-diols. Minor amounts of 30:1 and 32:1 1,15-alkanediols also were present. This distribution of alkanediols is closely similar to those in surficial sediments from the same region of the Baltic Sea (Morris, unpublished data) and in an S_1 sapropel from the Eastern Mediterranean (1,2).

Such comparability suggests a direct link between their occurrence in cyanobacterial blooms and in bottom sediments. Hence, the presence of alkanediols in sediments may be taken as an indication of lipid contributions from planktonic cyanobacteria. In addition, the abundance of these alkanediols in sediments illustrates the importance of such organisms in many marine environments. Significantly, the alkanediols only were present, if at all, at comparatively low levels of abundance in contemporary sediments from the Peru upwelling region and from Walvis Bay (Morris, unpublished data), and in Pleistocene sediments from Walvis Ridge (Brassell, unpublished data) (Table 1). These data suggest that the cyanobacteria that biosynthesize the alkanediols cannot compete and coexist with diatoms in such productive waters.

The recognition of a biological source for the alkanediols prompts consideration of the origin of the structurally similar hydroxyketones (11–13). It is plausible that the latter are oxidation products of the former, given the parallels (11,12) in their carbon number distributions. Alternatively, the hydroxyketones may be biosynthetic products generated by the cyanobacteria at other growth stages. In any event, the clear structural relationship between the alkanediols and the hydroxyketones (11) demonstrates that they are produced by a common biosynthetic pathway. Hence, the hydroxyketones also may be regarded as markers for cyanobacterial contributions to sediments.

The precise role of the alkanediols in cyanobacterial biochemistry or physiology is unclear. Their occurrence in *A. flos-aquae* in concentrations equivalent to those of sterols (Fig. 1) suggests that they may have a function in some aspect of metabolism or membrane structure. The specificity of the positions of their hydroxylation (*viz.* 1,13- and 1,15) may provide a clue to the pathways responsible for the biosynthesis of these unusual lipid components. Evidence for their biological importance comes from the frequency of their occurrence (Table 1) and their abundance in both marine (11,12) and lacustrine (17) sediments, which in turn supports the recent finding that cyanobacteria are major contributors to the aquatic biomass (14). In addition, the geological record of these compounds, as indicated by their presence in Eocene sediments (17), demonstrates that they have been biosynthesized actively for the greater part of the Tertiary (i.e. the last 50 Ma).

TABLE 2

Relative Abundance of Major Lipid Classes in Field Population of *A. flos-aquae*

Lipid class	%	Major compound
Carboxylic acids	40.5	Hexadecanoic acid
Alkanols	16.7	Docosanol
Sterols	15.1	Cholest-5-en-3β-ol
Alkanediols	13.0	Dotriaconta-1,15-diol
Phytol	10.0	Phytol
Hydrocarbons	2.0	Diploptene
Others	2.7	

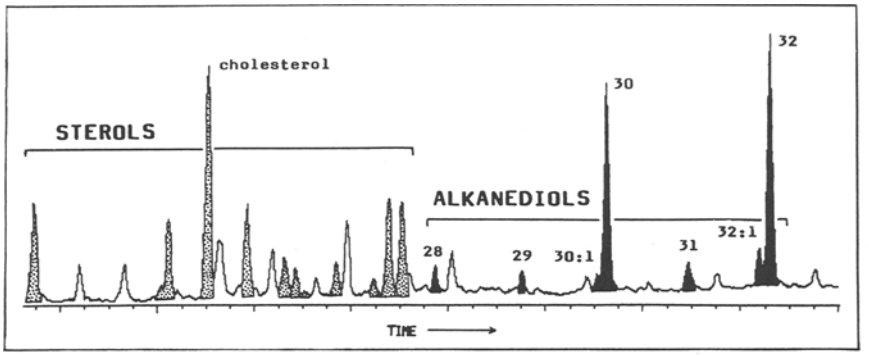

FIG. 1. Partial reconstituted ion chromatogram from GC-MS analysis of the neutral fraction (after trimethylsilylation) of *A. flos-aquae*. The region containing sterols and alkanediols is illustrated, and the carbon number of the latter compounds is shown. The sterols are C_{26}-C_{29} components with Δ^5, $\Delta^{5,22}$, $\Delta^{5,7,22}$ or $\Delta^{5,24(28)}$ unsaturation. No 5α(H)-stanols or 4-methylsterols were present. Hence, the recognition of such sterols in all the sediments containing alkanediols (11,12,15,16) is consistent with their presumed origin from dinoflagellates (3–5) and demonstrates that cyanobacteria are not the sole phytoplankton contributing sterols to the sediments.

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REFERENCES

1. Brassell, S.C., and Eglinton, G. (1983) in *Coastal Upwelling: Its Sediment Record* (Suess, E., and Thiede, J., eds.) pp. 545–574, Plenum Press, New York, NY.
2. Brassell, S.C., and Eglinton, G. (1986) in *Organic Marine Geochemistry* (Sohn, M., ed.) pp. 10–32, American Chemical Society, Washington, DC.
3. Boon, J.J., Rijpstra, W.I.C., de Large, F., de Leeuw, J.W., Yoshioka, M., and Shimizu, Y. (1979) *Nature 277*, 125–127.
4. de Leeuw, J.W., Rijpstra, W.I.C., Schenck, P.A., and Volkman, J.K. (1983) *Geochim. Cosmochim. Acta 47*, 455–465.
5. Robinson, N., Eglinton, G., Brassell, S.C., and Cranwell, P.A. (1984) *Nature 308*, 439–441.
6. Brassell, S.C., Wardroper, A.M.K., Thomson, I.D., Maxwell, J.R., and Eglinton, G. (1981) *Nature 290*, 693–696.
7. Holzer, G., Oro, J., and Tornabene, T.G. (1979) *J. Chromatogr. 186*, 795–809.
8. Volkman, J.K., Eglinton, G., Corner, E.D.S., and Sargent, J.R. (1980) in *Advances in Organic Geochemistry 1979* (Douglas, A.G., and Maxwell, J.R., eds.) pp. 219–227, Pergamon Press, Oxford, England.
9. Marlowe, I.T., Green, J.C., Neal, A.C., Brassell, S.C., Eglinton, G., and Course, P.A. (1984) *Br. Phycol. J. 19*, 203–216.
10. Simoneit, B.R.T., Chester, R., and Eglinton, G. (1977) *Nature 267*, 682–685.
11. de Leeuw, J.W., Rijpstra, W.I.C., and Schenck, P.A. (1981) *Geochim. Cosmochim. Acta 45*, 2281–2285.
12. Smith, D.J., Eglinton, G., and Morris, R.J. (1983) *Lipids 18*, 902–905.
13. Brassell, S.C., Eglinton, G., and Maxwell, J.R. (1981) *Init. Rep. DSDP 66*, 557–580.
14. Takahashi, M., Kikuchi, K., and Hara, Y. (1985) *Mar. Biol. 89*, 63–69.
15. ten Haven, H.L. (1986) *Organic and Inorganic Geochemical Aspects of Mediterranean Late Quaternary Sapropels and Messinian Deposits*, Ph.D. thesis, University of Utrecht, The Netherlands, Geologica Ultraiectina No. 46.
16. Comet, P.A. (1982) *The Use of Lipids as Facies Indicators*, Ph.D. thesis, University of Bristol, U.K.
17. Robinson, N. (1984) *Lipids in Lacustrine Environments*, Ph.D. thesis, University of Bristol, U.K.
18. Robinson, N., Cranwell, P.A., Eglinton, G., Brassell, S.C., Sharp, C.L., Gophen, M., and Pollingher, U. (1986) in *Advances in Organic Geochemistry 1985* (Leythaeuser, D., and Rullkotter, J., eds.) pp. 733–742, Pergamon Press, Oxford, England.
19. de Leeuw, J.W. (1986) in *Organic Marine Geochemistry* (Sohn, M., ed.) pp. 33–61, American Chemical Society, Washington, DC.
20. Thomson, I.D., Brassell, S.C., Eglinton, G., and Maxwell, J.R. (1982) *Init. Rep. DSDP 64*, 913–919.
21. Davis, P.S. (1957) *CSIRO Div. Fish. Oceanogr. Tech. Rep. No. 7*, 1–8.
22. Morris, R.J., McCartney, M.J., Joint, I.R., and Robinson, G.A. (1985) *J. Exp. Mar. Biol. Ecol. 86*, 151–170.
23. Folch, J.M., Lees, M., and Stone-Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497–509.
24. Culkin, F., and Morris, R.J. (1969) *Deep Sea Res. 16*, 109–116.

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High Density Lipoproteins from Bovine Plasma and Follicular Fluid Do Not Possess a High Affinity for Glycosaminoglycans

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Possible interactions between glycosaminoglycans and high density lipoproteins (HDL) in plasma and follicular fluid were examined. Total lipoproteins (d < 1.21 g/ml) were obtained from plasma of five Holstein cows by ultracentrifugation and fractionated by gel filtration. Every other fraction from the HDL peak or fractions corresponding to the base and ascending portion of the HDL peak were composited and applied to a heparin-Sepharose affinity chromatography column. Elution profiles from both composites showed a peak that did not bind to the column that contained HDL devoid of apolipoprotein-E as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining and immunoblot analysis. Elution of lipoproteins from the ascending portion of the HDL peak resulted in a second minor peak eluting at 0.35 M NaCl, which was low density lipoprotein (LDL) contamination. Lipoproteins (d < 1.21 g/ml) isolated from follicular fluid obtained from small, medium or large follicles also were subjected to heparin-Sepharose affinity chromatography. Two peaks were observed, one corresponding to the lipoprotein that did not bind to the column, the other eluted at 0.5 M NaCl and accounted for less than 2% of the protein applied. The second peak did not contain apolipoprotein-E or LDL. Bovine follicular fluid glycosaminoglycans (GAG) were isolated and subjected to HDL-Sepharose affinity chromatography. Less than 2% of the total GAG bound to the HDL column. Therefore, HDL in bovine specimens did not interact appreciably with heparin or GAG isolated from follicular fluid.
Lipids 23, 269-274 (1988).

Lipoproteins are a source of cholesterol for ovarian tissue. In bovine luteal and granulosa cell cultures, high density lipoproteins (HDL) and low density lipoproteins (LDL) can maintain progesterone production in serum-free media (1,2). In porcine, humans and rats, the preferential source for steroidogenesis by ovarian tissue is the same lipoprotein fraction that accounts for the highest proportion of cholesterol in plasma. It may be expected that bovine ovarian tissue utilizes HDL sterol because its total concentration of plasma cholesterol is 15 times greater than LDL. HDL also is the predominant lipoprotein in bovine, porcine and human follicular fluid (1,3,4). HDL can be accumulated into steroidogenic tissue by several pathways. One pathway, via the B, E receptor originally described by Goldstein et al. (5), is specific for HDL-bearing apolipoprotein-E, an arginine-rich glycoprotein. A second pathway also is receptor-dependent and specific for HDL devoid of apolipoprotein-E (6). Recently, Nestler et al. (7) demonstrated HDL binding that did not require a high-affinity binding site.

Glycosaminoglycans (GAG) also are constituents of bovine follicular fluid (8). They are polysaccharides composed of repeating disaccharide units and usually are attached to a protein core. In bovine follicular fluid, their concentrations decrease with increasing follicular size (9) and are negatively correlated with estradiol 17-β concentrations (10). GAG can bind apolipoprotein-B and E in nonruminants (11). Apolipoprotein-B and E are rich in basic amino acids that bind to negatively charged sulfate groups in GAG. Sulfation of GAG seems crucial for interaction because desulfated GAG do not interact with lipoproteins (12).

Contradictory reports can be found in the literature regarding the presence of apolipoprotein-E in bovine plasma. Forte et al. (13) demonstrated the presence of an apolipoprotein at the same molecular weight as nonruminant apolipoprotein-E (35,000 daltons), while other researchers (14,15) did not find any apolipoproteins in that molecular weight range. Rat granulosa cells in vitro produce apolipoprotein-E (16). Both cyclic adenosine monophosphate (cAMP) and follicle-stimulating hormone (FSH) stimulate apolipoprotein production. Thus, the rat granulosa cells might be modifying follicular fluid lipoproteins. If apolipoprotein-E is needed for cholesterol binding to rat granulosa cells, the cells could be generating a receptor ligand to increase cellular cholesterol uptake.

This study was designed to determine if bovine plasma or follicular fluid contained apolipoprotein-E and if there was an interaction between GAG and HDL from blood or follicular fluid. If HDL cholesterol is an important precursor for steroidogensis, GAG-HDL interactions may influence ovarian function.

MATERIALS AND METHODS

Plasma lipoprotein isolation. Two hundred fifty ml of blood were obtained from the jugular veins of five Holstein cows at various stages of lactation. The blood was collected in a flask containing disodium ethylene diaminetetraacetate (Na_2EDTA), sodium azide (NaN_3), gentamycin sulfate, ε-amino caproic acid and phenylmethylsulfonyl fluoride to provide final concentrations in blood of 0.1, 0.05, 0.005, 0.13 and 0.26%, respectively. Plasma was obtained by low-speed centrifugation and was adjusted to a density of 1.21 gm/ml with solid potassium bromide and centrifuged at 35,000 rpm (Beckman 50.2 Ti rotor) at 20 C for 18 hr.

Total lipoproteins were collected by aspiration and concentrated to a volume of 10 ml by ultrafiltration (50 ml Amicon stirred cell, Model 8050, Diaflow Membrane: YM10). The lipoprotein sample then was subjected to gel filtration chromatography. Bio-Gel A5-M, 200-400 mesh (Bio-Rad Laboratories, Richmond, CA) was packed to a bed height of 92 cm in a K 26/100 column (Pharmacia, Uppsala, Sweden). Samples were applied to the column

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Abbreviations: cAMP, cyclic adenosine monophosphate; FSH, follicle-stimulating hormone; GAG, glycosaminoglycans; HDL, high density lipoproteins; LDL, low density lipoproteins; Na_2EDTA, disodium ethylenediaminetetraacetate; NaN_3, sodium azide; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; VLDL, very low density lipoprotein.

and eluted at 20 C with 0.15 *M* sodium chloride (NaCl) containing Na_2EDTA, NaN_3, gentamycin sulfate, ε-amino caproic acid and phenylmethylsulfonyl fluoride at final concentrations of 0.01, 0.2, 0.02, 0.13 and 0.26%, respectively (pH 8.0).

Ninety 5-ml fractions were collected at an elution rate of 17 ml/hr. Protein was monitored at 280 nm. Cholesterol (Sigma Cholesterol 20, St. Louis, MO) and protein (Pierce BCA Lowry Protein Assay, Rockford, IL) concentrations were measured on the fractions in the HDL peak. Fractions of interest were composited and concentrated by ultrafiltration (10 ml Amicon stirred cell Model 12, Diaflo Membrane YM10, Danvers, MA) and protein measured according to Bradford (17) before affinity chromatography.

Follicular fluid lipoprotein isolation. Follicular fluid was aspirated from small (<6 mm), medium (6–10 mm) or large (11–20 mm) follicles within 30 min of slaughter (Packerland Packing, Green Bay, WI). Granulosa cells were removed by centrifugation (2,575 × g). Protease inhibitors were added at concentrations of 0.2% NaN_3, 0.02% gentamycin sulfate, 0.13% ε-amino caproic acid, and 0.26% phenylmethylsulfonyl fluoride. Fluid was frozen and stored at −20 C. Fluid was thawed, adjusted to a density of 1.21 g/ml with solid potassium bromide and centrifuged at 35,000 rpm (Beckman 50.2 Ti rotor) for 18 hr at 20 C. Total lipoproteins were aspirated, concentrated by ultrafiltration and resuspended in affinity column starting buffer (2 mM phosphate buffer, 50 mM NaCl, 0.02% NaN_3, pH 7.4).

Affinity chromatography. Affinity chromatography was performed as described (18). Briefly, 10 mg of lipoprotein were applied to the heparin-Sepharose affinity column (1.5 × 9 cm, heparin Sepharose CL-6B, Pharmacia, Uppsala, Sweden) after equilibration with starting buffer. The column was washed with 40 ml starting buffer to elute unbound proteins. A 120 ml, 0.05 to 1.5 M NaCl linear gradient was used to elute bound proteins (0.3 ml/min). Three ml fractions were collected, and the absorbance of light at 280 nm and conductivity of each fraction were monitored. SDS-PAGE followed by silver staining (19) was performed to determine the apolipoprotein profile of lipoproteins contained within each peak.

HDL-Sepharose chromatography. Columns were prepared by linking 80 mg of HDL to Sepharose (1.5 × 4.5 cm, CnBr activated 4B, Pharmacia, Uppsala, Sweden) by the procedure of Hay and Getz (20). Follicular fluid GAG were obtained as described (21) from small (<6 mm) or large (11–20 mm) follicles. Follicular fluid GAG concentrations were calculated with reference to a standard curve of commercially available heparin (Calbiochem, La Jolla, CA). Three mg of GAG isolated from fluid of small or large follicles were applied to the HDL-Sepharose affinity column. Twenty-five ml of starting buffer (10 mM Tris, 10 mM $CaCl_2$, 0.02% NaN_3, pH 8.0) was used to elute unbound GAG. A 40 ml, 0 to 2 M linear gradient was used to elute bound proteins (flow rate = 0.2 ml/min). One ml fractions were collected, and GAG were quantitated by gel filtration high performance liquid chromatography (HPLC; I-125 column, Waters Associates, Framingham, MA [22]). Conductivity of each fraction was measured to determine the molarity at which GAG eluted.

Immunoblot analysis. Immunoblot analysis was performed according to (23) using antihorseradish peroxidase as the second antibody. Antisera to human and porcine apolipoprotein-E were donated by Walter McConathy, Oklahoma Medical Research Foundation and Alan D. Attie, Department of Biochemistry, University of Wisconsin, respectively. Antiserum to porcine apolipoprotein-E was developed in chickens and antiserum to human apolipoprotein-E was developed in rabbits. Human VLDL was a gift from Attie.

RESULTS

A representative elution profile obtained when fractionating total lipoproteins by gel filtration is shown in Figure 1. The three peaks corresponded to very low density lipoproteins (VLDL), LDL, and HDL; HDL and LDL could not be separated completely. Cholesterol and protein were measured on the fractions containing HDL; there was a positive relation between lipoprotein particle size and cholesterol to protein ratio (Fig. 1). Fractions containing the largest HDL particles had minor LDL contamination that probably accounted for a small proportion of the increase in cholesterol to protein ratio. However, it was reported that the least dense HDL particles have a higher cholesterol content (24,25).

To avoid LDL contamination, lipoprotein fractions 58 to 70 from the HDL peak represented by the solid line in Figure 1 were composited and applied to the heparin-Sepharose column. Two peaks eluted, one corresponding to lipoprotein that did not bind to the column, the other eluting at 0.3 M NaCl. SDS-PAGE revealed that lipoproteins that did not bind to the column contained predominantly a 28,000 dalton protein (data not shown). Insufficient protein was present in the second peak to be analyzed by SDS-PAGE and silver staining of proteins.

Cholesterol-rich lipoproteins from fractions 53 to 58 represented by the dotted line (Fig. 1) also were applied to the heparin-Sepharose column. The chromatogram is shown in Figure 2. Peak A represents lipoproteins that did not bind to the column and lipoproteins in peak B eluted at 0.3 M NaCl. As noted with the low cholesterol HDL fraction, proteins in peak A contained substantial apolipoprotein A-I (28,000 daltons), which is indicative of HDL (Fig. 3). In addition, a 12,000 dalton protein of unknown identity was enriched in the cholesterol-rich HDL fraction. Whether this is a native protein or protein fragment is not known. Peak B obtained after affinity chromatography of cholesterol-rich lipoproteins contained small amounts of A-I. Bovine LDL isolated by gel filtration previously has been shown to contain apolipoprotein A-I (Grummer et al., unpublished data). Also observed were large molecular weight proteins that are probably B apolipoproteins. Bovine LDL isolated by gel filtration show multiple high molecular weight proteins when silver stain (but not coomassie blue stain) is used with SDS-PAGE, and these proteins do not appear to be due to proteolytic cleavage of protein after sampling blood (Grummer et al., unpublished data). Thus, peak B was considered to be LDL contamination of cholesterol-rich HDL. As recently reported (26), heparin-Sepharose affinity chromatography separates bovine HDL from LDL. Apolipoprotein-E has been reported to have a molecular weight of 35,000 daltons in nonruminants (27). From our data, bovine HDL appears to lack a protein of similar size.

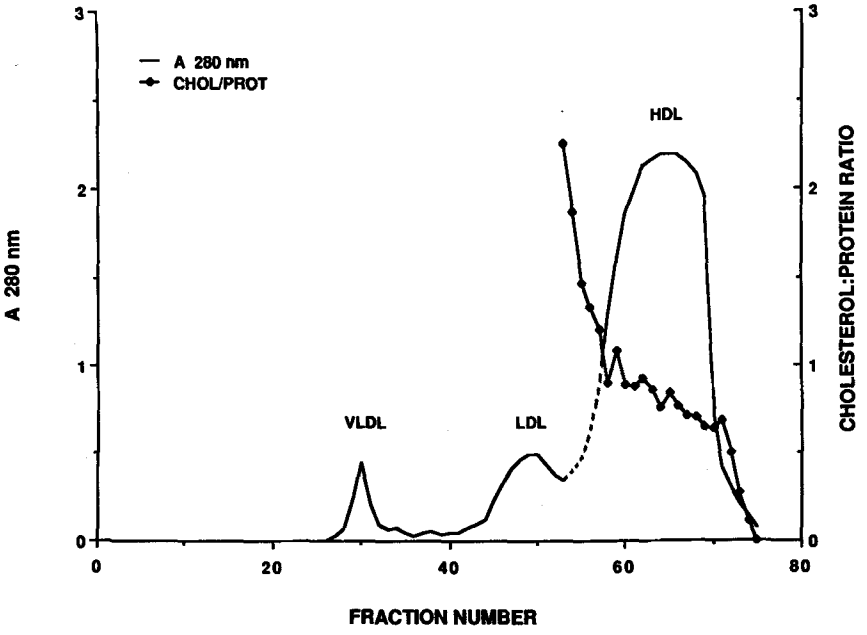

FIG. 1. Elution profile obtained by subjecting total plasma lipoproteins to gel filtration chromatography. Protein in each fraction was monitored at 280 nm (—— or – – – –). Fractions represented by (– – – –) are defined as containing cholesterol-rich HDL. Cholesterol and protein were assayed in each HDL fraction to determine cholesterol to protein ratios (——◆——).

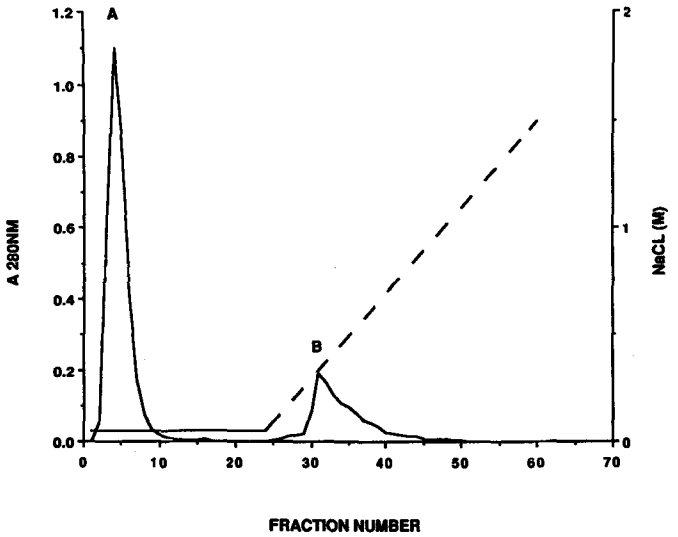

FIG. 2. Elution profile obtained by heparin-Sepharose affinity chromatography of cholesterol-rich fractions that are represented by the dotted line (– – – –) in Figure 1. Forty ml of equilibrating buffer was applied followed by 120 ml linear gradient from 50 mM to 1.5 *M* NaCl. Protein (A 280 nm, ——) and conductivity (– – – –) of each fraction was monitored. Peak A represents unbound lipoprotein while protein in peak B eluted at 0.3 *M* NaCl.

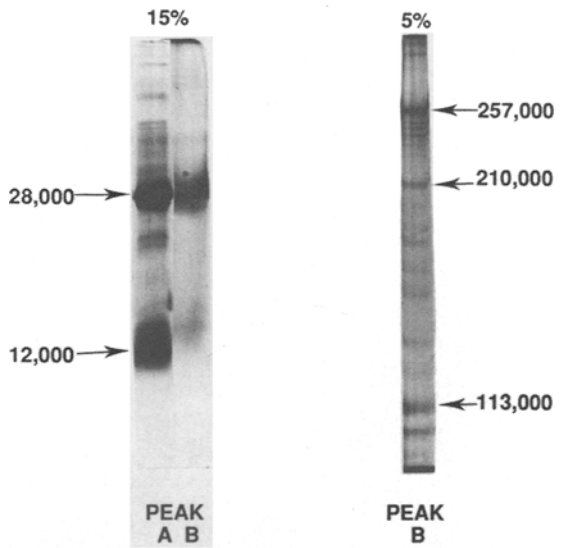

FIG. 3. SDS-PAGE gels (5 and 15% acrylamide) of proteins eluting in peak A or B (Fig. 2) during heparin-Sepharose chromatography of cholesterol-rich HDL.

Lipoproteins (d < 1.21 g/ml) isolated from follicular fluid of small, medium or large follicles also were analyzed by heparin chromatography to determine if follicular and plasma lipoproteins exhibited chemical differences. Lipoproteins isolated from the fluid of small follicles were applied and eluted from the heparin-Sepharose column; two peaks resulted (Fig. 4). Peak A represents lipoproteins that did not bind to the column, while lipoproteins within peak B eluted at 0.5 M NaCl. Heparin-Sepharose affinity chromatography of lipoproteins from medium and large follicles resulted in similar elution profiles. SDS-PAGE followed by silver staining of apolipoproteins in

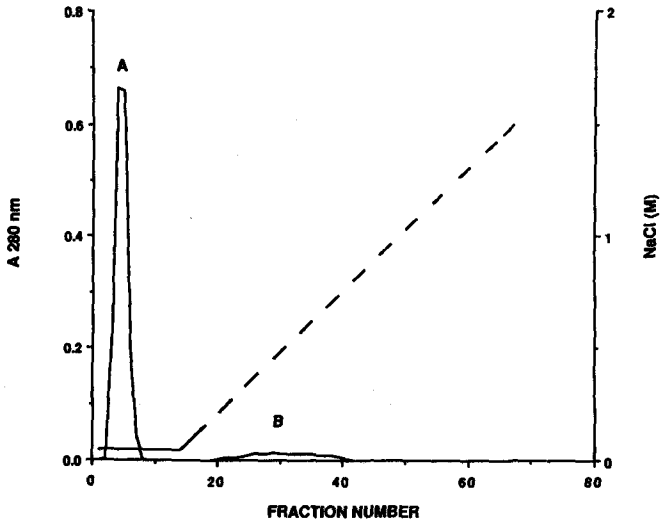

FIG. 4. Elution profile obtained when subjecting lipoproteins isolated from fluid of small follicles (<6 mm) to heparin-Sepharose affinity chromatography. Forty ml of equilibrating buffer was applied followed by a 120 ml linear gradient from 50 mM to 1.5 *M* NaCl. Protein (A 280, ——) and conductivity (– – – –) of each fraction was monitored. Protein in peak A did not bind to the column, while protein in peak B eluted at 0.5 *M* NaCl.

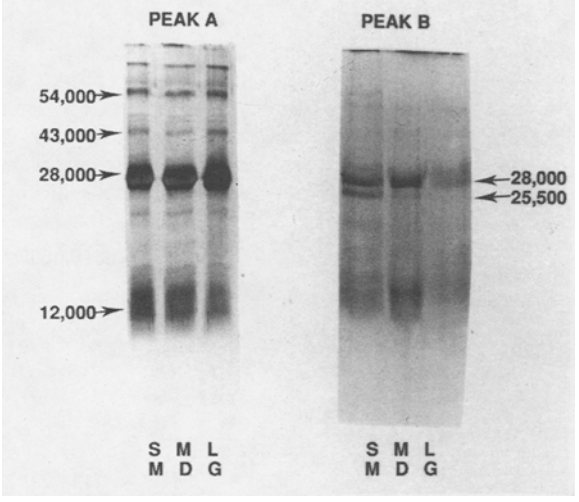

FIG. 5. SDS-PAGE (15% acrylamide) of proteins eluting in peak A or B (Fig. 4) during heparin-Sepharose chromatography of lipoproteins isolated from the fluid of small follicles.

the unbound fraction showed they contained an apolipoprotein profile similar to plasma HDL. The lipoproteins that eluted at 0.5 M NaCl contained an additional protein of unknown identity at 25,500 daltons, which decreased in intensity with increasing follicle size (Fig. 5). Large molecular weight B apolipoproteins were not observed (5% acrylamide, gels not shown) in the d < 1.21 g/ml fraction, therefore, HDL appears to be the only lipoprotein in follicular fluid. Furthermore, the lipid and protein composition of the d < 1.21 g/ml fraction averaged 28.3% phospholipid, 29.8% cholesterol (total), nondetectable triglyceride and 41.9% protein, which is

TABLE 1

Percentage of Total HDL Protein Isolated from Follicular Fluid that Bound to Heparin-Sepharose Affinity Columns

Follicle size	Mean[a]	SEM
Small	1.9	0.4
Medium	1.4	0.7
Large	1.0	0.2

[a]From three replicates.

TABLE 2

Percentage of Total GAG Isolated From Follicular Fluid that Bound to the HDL-Sepharose Affinity Column

Follice size	Molarity of NaCl in fractions			
	0-1 M		1-2 M	
	Mean[a]	SEM	Mean	SEM
	%			
Small	2.0	0.2	0.39	0.19
Large	3.3	1.9	0.23	0.03

[a]From two replicates.

similar to bovine plasma HDL composition (28), particularly smaller, more dense HDL that are more likely to penetrate the blood-follicular fluid barrier. Less than 2% of total protein applied to heparin columns bound (Table 1), indicating little interaction between follicular fluid lipoproteins and commercially available heparin linked to Sepharose.

Heparan sulfate and chondroitin sulfate are the two predominant GAG in follicular fluid (29), not the heparin that is linked commercially to Sepharose. Thus, the extent to which naturally occurring GAG isolated from bovine follicular fluid bind to a HDL-Sepharose affinity column was examined. A representative elution profile obtained when GAG isolated from follicular fluid was applied to the HDL-Sepharose affinity column is shown in Figure 6. In Table 2, the percentage of total GAG that bound to HDL-Sepharose and eluted from 0 to 1 M NaCl and 1 to 2 M NaCl are shown. Greater than 95% of the GAG did not bind to the column. Therefore, using homologous preparations, very little interaction between bovine follicular fluid GAG and HDL occurred. Purified dermatan sulfate (a subclass of chondroitin sulfate and most abundant follicular fluid GAG) from large follicles or commercial heparin was applied to a LDL-Sepharose column that was prepared in the same manner as the HDL-Sepharose column. Sixteen percent of the dermatan sulfate from large follicles and 67% of the heparin bound to the column and was eluted with 2 M NaCl. GAG bind to LDL via apolipoprotein-B (11), therefore, the methodology we employed should have been valid for demonstrating apolipoprotein-(E)-GAG interaction.

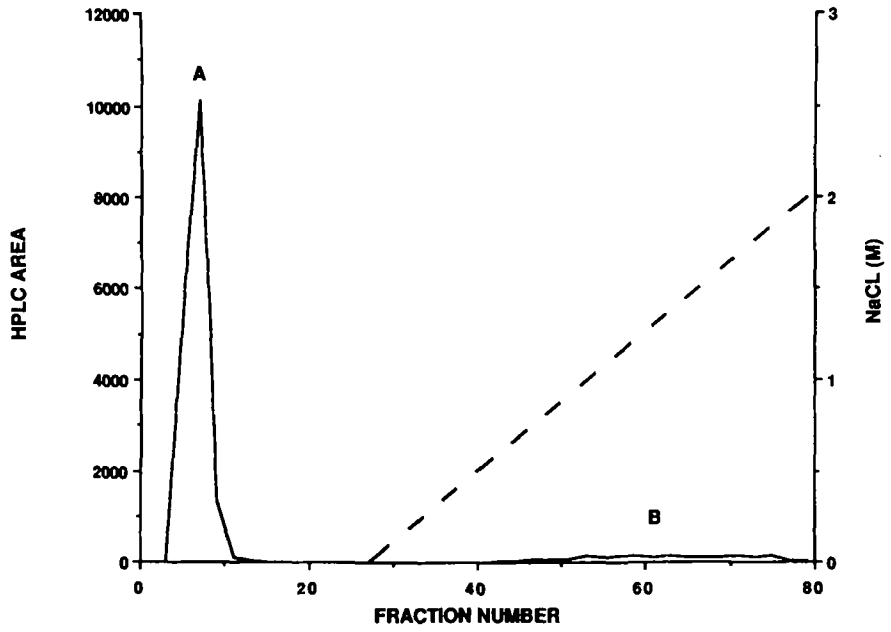

FIG. 6. Elution profile obtained when subjecting GAG isolated from fluid of small follicles to HDL-Sepharose affinity chromatography. Twenty-five ml of equilibrating buffer was applied followed by a 40 ml linear gradient from 0 to 2 *M* NaCl. GAG content (HPLC Area, Waters I-125, ——) and conductivity (– – – –) of each fraction was monitored. GAG in peak A did not bind to the column while GAG in peak B bound from 0.5 to 2 *M* NaCl.

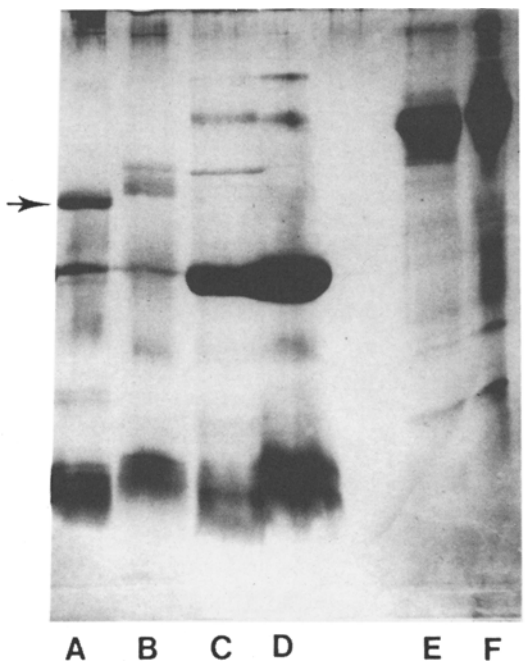

FIG. 7. SDS-PAGE gel (15% acrylamide) of: A-porcine plasma VLDL, B-bovine plasma VLDL, C-bovine follicular fluid HDL, D-bovine plasma HDL, E-follicular fluid subnatant from total lipoprotein spin (density > 1.21 g/ml), F-plasma subnatant from total lipoprotein spin (density > 1.21 g/ml). Arrow indicates porcine VLDL protein immunoreactive with anti-apolipoprotein-E sera.

Although steps were taken to minimize loss of apolipoprotein-E. (single slow-speed ultracentrifugation and gel filtration), some may have occurred during isolation. Therefore, the subnatant obtained after isolation of total lipoproteins from plasma and follicular fluid was subjected to SDS-PAGE. Those fractions as well as plasma and follicular fluid HDL generated from the same samples all appeared void of apolipoprotein-E (Fig. 7). Immunoblot analysis using antisera specific for either porcine or human apolipoprotein-E (generated in chickens and rabbits, respectively) demonstrated that those fractions as well as bovine VLDL were deficient in apolipoprotein-E or that the antibody did not cross react with bovine apolipoprotein-E. However, both porcine and human VLDL apolipoprotein-E were precipitated with either antiserum.

DISCUSSION

Results from this study confirm other reports that HDL devoid of apolipoprotein-E does not interact with commercially available GAG (30,31). Results from studies examining if bovine plasma contains apolipoprotein-E are contradictory (13,14). Other species such as the rat have cholesterol-rich particles (HDL_c) below a density of 1.063 g/ml that contain apolipoprotein-E (32). It was hypothesized that bovine HDL would contain apolipoprotein-E since bovine plasma contains cholesterol-rich, α-migrating lipoproteins having a density less than 1.063 g/ml (25,28,33).

Cordle et al. (26) applied a combined bovine LDL-HDL fraction to a heparin-Sepharose column and did not observe apolipoprotein-E in the bound lipoprotein fraction eluting from the column. Since a combined LDL-HDL fraction was used, insufficient apolipoprotein-E-rich HDL may have been applied to the column. Additionally, a relatively insensitive stain, Coomassie blue, was used for detecting proteins after SDS-PAGE of eluted lipoproteins from the heparin-Sepharose affinity column. However,

in agreement with Cordle et al. (26), our results from heparin-Sepharose affinity chromatography of cholesterol-rich plasma HDL, coupled with use of silver stain for protein detection and immunoblot analysis following SDS-PAGE of lipoprotein fractions, indicated the absence of apolipoprotein-E in bovine plasma HDL. HDL obtained from bovine follicular fluid also showed limited interaction with heparin-Sepharose. Thus, bovine plasma and follicular fluid HDL do not appear to contain a lipid or apolipoprotein moiety that binds to heparin.

Since GAG are a very heterogeneous class of compounds (34), an additional experiment was performed to determine if naturally occurring GAG isolated from follicular fluid (chondroitin sulfate and heparan sulfate; 29) possessed an affinity for HDL, permitting a homologous comparison that might reflect physiological interactions. This is the first study, to our knowledge, to examine if GAG found in follicular fluid interact with HDL. However, little interaction was seen between these GAG and HDL-Sepharose (Table 2). Therefore, if GAG are affecting steroidogenesis, it is not likely due to them binding HDL and preventing delivery of cholesterol to granulosa cells. The possibility remains that GAG affect lipoprotein metabolism at the cellular level since LDL degradation is inhibited in a dose-dependent manner by increased concentrations of GAG in porcine granulosa cell cultures (35).

Indirect evidence from this study indicates that the bovine granulosa cell probably is not producing appreciable quantities of apolipoprotein-E and sequestering it into the follicular fluid HDL. The apolipoprotein-E that was produced in rat granulosa cell cultures was found at a density less than 1.21 g/ml (16). The researchers hypothesized it was attached to a lipoprotein but did not provide any evidence to confirm this. HDL appears to be the only class of lipoproteins found in bovine follicular fluid ([1] Fig. 5) and would be the only exogenous source of cholesterol for progesterone production by granulosa cells. Bovine granulosa cells produce copious quantities of progesterone prior to ovulation (36). Thus, before vascularization of the granulosa cell layer, tremendous amounts of substrates are necessary for progesterone production. HDL cholesterol found in follicular fluid is a logical substrate. Without apolipoprotein-E on the HDL molecule, bovine granulosa cell receptors must recognize a different HDL constituent. The characteristics of those receptors and the possibility that GAG affect their function need to be determined.

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REFERENCES

1. Savion, N., Laherty, R., Cohen, D., Lui, G., and Gospodarowicz, D. (1982) *Endocrinology 110,* 13-23.
2. O'Shaughnessy, P.J., and Wattes, D.C. (1985) *J. Reprod. Fert. 74,* 425-432.
3. Chang, S.C.S., Jones, J.D., Ellefson, R.D., and Ryan, R.J. (1976) *Biol. Reprod. 15,* 321-328.
4. Enk, L., Crona, N., Olsson, J.H., and Hillensjo, T. (1986) *Acta Endocrin. 111,* 558-562.
5. Goldstein, J.L., Basu, S.K., Brunschede, G.Y., and Brown, M.S. (1976) *Cell 7,* 85-95.
6. Hwang, J., and Menon, K.M.J. (1983) *J. Biol. Chem. 258,* 8020-8027.
7. Nestler, J.E., Chacko, G.K., and Strauss, J.F. (1985) *J. Biol. Chem. 260,* 7316-7321.
8. Grimek, H.J., and Ax, R.L. (1982) *Biochem. Biophys. Res. Commun. 104,* 1401-1406.
9. Bellin, M.E., and Ax, R.L. (1984) *Endocrinology 114,* 428-433.
10. Bushmeyer, S.M., Bellin, M.E., Brantmeier, S.A., Boehm, S.K., Kubajak, C.L., and Ax, R.L. (1985) *Endocrinology 117,* 879-885.
11. Mahley, R.W., Weisgraber, K.H., and Innerarity, T.L. (1979) *Biochim. Biophys. Acta 575,* 81-91.
12. Vijayagopal, P., Srinivasan, S.R., Radhakrishnamurthy, B., and Berenson, G.S. (1981) *J. Biol. Chem. 256,* 8234-8241.
13. Forte, T.M., Nordhausen, R.W., Nichols, A.V., Endemann, G., Miljanich, P. and Bell-Quint, J.J. (1979) *Biochim. Biophys. Acta 573,* 451-463.
14. Jonas, A. (1975) *Biochim. Biophys. Acta 393,* 460-470.
15. Lim, C.J., and Scanu, A.M. (1976) *Artery 2,* 483-496.
16. Driscoll, D.M., Schreiber, J.R., Schmit, V.M., and Getz, G.S. (1985) *J. Biol. Chem. 260,* 9031-9038.
17. Bradford, M.M. (1976) *Anal. Biochem. 72,* 248-254.
18. Shelburne, F.A., and Quarfordt, J.H. (1977) *J. Clin. Invest. 60,* 944-950.
19. Grummer, R.R., Hurley, W.L., Davis, C.L. and Meacham, C.A. (1986) *J. Dairy Sci. 69,* 2083-2090.
20. Hay, R.V., and Getz, G.S. (1979) *J. Lipid Res. 20,* 334-351.
21. Bellin, M.E. (1986) *Synthesis, Distribution, and Composition of Glycosaminoglycans during Folliculogenesis,* M.S. Thesis, Univ. of Wisconsin-Madison, WI.
22. Ax, R.L., and Ryan, R.J. (1979) *Biol. Reprod. 20,* 1123-1132.
23. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. 76,* 4350-4354.
24. Puppione, D.L. (1978) *J. Dairy Sci. 61,* 651-659.
25. Raphael, B.C., Dimick, P.S., and Puppione, D.L. (1973) *J. Dairy Sci. 56,* 1025.
26. Cordle, S.R., Clegg, R.A., and Yeaman, S.J. (1985) *J. Lipid Res. 26,* 721-725.
27. Rall, S.C., Weisgraber, K.H., and Mahley, R.W. (1982) *J. Biol. Chem. 257,* 4171-4178.
28. Puppione, D.L., Kunitake, S.T., Toomey, M.L., Loh, E., and Schumaker, V.W. (1982) *J. Lipid Res. 23,* 371-379.
29. Ax, R.L., Bellin, M.E., and Grimek, H.J. (1985) in *Proceedings of the Fifth Ovarian Workshop* (Toft, D.O. and Ryan, R.J., eds.) pp. 451-480, American Oil Chemists' Society, Champaign, IL.
30. Iverius, P.H. (1972) *J. Biol. Chem. 247,* 2607-2613.
31. Christie, M.J., Gwynne, J.T., and Strauss, J.F. (1981) *J. Steroid Biochem. 14,* 671-678.
32. Zannis, V.I., and Breslow, J.L. (1982) *Mol. Cell. Biochem. 4,* 3-20.
33. Grummer, R.R., Davis, C.L., and Hegarty, H.M. (1983) *Lipids 18,* 795-802.
34. Lindahl, U., and Hook, M. (1978) *Ann. Rev. Biochem. 47,* 385-417.
35. Bellin, M.E., Veldhuis, J.D., and Ax, R.L. (1987) *Biol. Reprod. 37,* 1179-1184.
36. Dieleman, S.J., and Blankenstein, O.M. (1984) *J. Reprod. Fert. 72,* 487-494.

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Effects of Relative Humidity on Lipid Autoxidation in a Model System

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A new model system was developed for the study of autoxidation of thin films of neat lipid and the effect of relative humidity on the oxidation reaction. In the model system, the surface-to-volume ratio of lipid was large and measureable, and the relative humidity (RH) and oxygen partial pressure were controlled. Methyl linoleate, oxidized at six different RH as a thin film in an atmosphere of pure oxygen, exhibited a maximum rate of oxidation at 32% RH and minimum rates at 0% and 100% RH. The rates of oxygen uptake, determined manometrically, were linear and reproducible at all six RH. The maximum rate at 32% RH was attributed to solvation and stabilization of the propagation transition state by water. Increasing the RH beyond 32% resulted in solvation of the peroxy radical, sterically hindering the radical from entering the propagation transition state.

Lipids 23, 275-279 (1988).

Free radical-mediated lipid autoxidation is the major cause of quality deterioration (rancidity) and decreased nutritional quality and safety in lipid-containing foods (1,2). The importance of lipid oxidation extends beyond the economic considerations associated with food. The biological problems associated with lipid peroxidation include damage to membranes, enzymes, vitamins and cell metabolism (3,4). Lipid autoxidation has been implicated in some species in several normal and abnormal processes including aging, cancer, hemolytic anemia, reproductive dysfunction and lung damage, among many others (5,6).

The complex and varying make-up of biological material causes difficulty in pinpointing any single element, compound or condition as the sole cause of a given effect. It is essential to have a model system that can be used to study the interaction of lipids with water, proteins, carbohydrates, and other biological molecules and the affect of these components on the rate and mechanism of lipid autoxidation. For this reason, a model system that can be used to evaluate these interactions was developed.

The primary goal of this study is to investigate and better understand the effect of water on the rate of lipid autoxidation. A unique relationship exists between the moisture content of biological material such as food and the rate of lipid autoxidation. Water exhibits both a pro- and antioxidant effect on lipid oxidation in these systems (1). To gain a better understanding of the relationship between moisture content and lipid autoxidation, the effect of relative humidity on the oxidation of a thin film of methyl linoleate is investigated.

EXPERIMENTAL PROCEDURES

The model consists of a thin film of lipid on a chemically modified glass support. Silica gel has been used in many studies as a support for lipid films (7-10), but the surface area of the irregularly shaped silica gel is difficult to determine. A 22 mm diameter circular glass coverslip was used as the support. The thickness of the lipid film can be varied to simulate the physical conditions of many different biological materials. For example, a thick film can be used to simulate the surface of bulk fats, a thin film can be used to simulate fat as it occurs in some foods, and a monolayer can be used to simulate membranes.

Acid treatment of glass coverslips. The glass coverslips were treated with hydrofluoric acid to form a thin layer of silica gel on the surface. The disks were treated by submerging them in concentrated hydrofluoric acid for four to five sec, rinsing with distilled water, neutralizing in 30% sodium hydroxide and rinsing thoroughly with distilled water. Care should be taken not to allow skin contact with concentrated hydrofluoric acid, and it should be stored in plastic containers. The treated disks were dried in a desiccator containing phosphorous pentoxide under nitrogen for a minimum of 48 hr and were stored in the desiccator until used. Later, it was discovered that a more consistent lipid film would form on the disks if the treated glass disks were baked in a 140 C oven overnight and used directly from the oven after a few minutes of cooling. Methyl linoleate formed an even layer without beading on the treated glass disks.

Reaction vessel. A modified Warburg Respirometer was used to monitor oxygen consumption during the reaction. The reaction vessel (Fig. 1) and manometer were modified by adding a 29/32 standard taper glass joint (Schott Glass KPV 29) to accept the 22 mm glass disk. The main chamber contained a glass pedestal to suspend the disk over a reservoir (2 ml) of water, desiccant or salt slurry for relative humidity (RH) control. A sidearm port with detachable septum was included to aid in purging the system with oxygen. The assembled reaction flask was

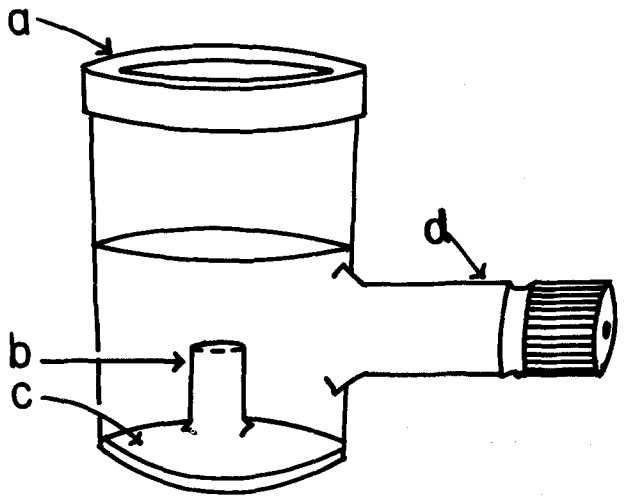

FIG. 1. Modified Warburg reaction flask: a) wide mouth to accept 22 mm disk; b) pedestal to support disk above reservoir; c) reservoir for saturated salt slurry, water or desiccant; and d) sidearm port for flushing system with oxygen.

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Abbreviation: RH, relative humidity.

immersed in a constant temperature water bath (37 C ± 0.5 C). The modified flask was calibrated according to standard Warburg procedures (11).

Humidity control and thin film preparation. The effect of relative humidity on autoxidation of a thin film of methyl linoleate was used to evaluate the reproducibility of the thin film model and the effect of water on lipid autoxidation. Methyl linoleate (Sigma Chemical Co., St. Louis, MO) was purified by the method of Privett and Blank (12) and stored under nitrogen at −20 C. The lipid was diluted with benzene to ca. 10 mM, and the equivalent of 2.7 mg of lipid was spread on an acid-treated glass disk and the benzene evaporated. The lipid on the disk was equilibrated to the desired relative humidity by placing it in a desiccator with the appropriate saturated salt slurry (13) or desiccant under nitrogen in the dark. If the disk was to be dried to 0% RH, it was left in the phosphorus pentoxide desiccator for a minimum of 48 hr. After equilibration, the disk was placed in the modified Warburg reaction flask on the pedestal above a 2 ml reservoir of the same saturated slurry, water or desiccant ($CaSO_4$) as needed to maintain the desired RH. The system was flushed with oxygen that had been adjusted to the desired RH by bubbling the oxygen through the appropriate salt slurry or desiccant. The rate of oxygen uptake was monitored manometrically at two- to four-hr intervals.

Reproducibility. The reproducibility of the new model system was verified statistically. Six independent runs at 72% relative humidity and five independent runs at 100% RH were measured, and an analysis of variance of beta regression coefficient (slope) was performed on each treatment. Within each treatment, the six (or five) runs were placed randomly in two groups and the F test used with the null hypothesis showed that there was no difference.

Tritiated water studies. The amount of water bound to a thin film of lipid was estimated by incubation with tritiated water. Tritiated water (1 mCi/ml, New England Nuclear, Boston, MA) was diluted with double glass-distilled water to a specific activity of 0.5 mCi/ml and placed in a ground glass weighing dish. Acid-washed glass wool under a perforated teflon disk supported the glass disks above the water. The perforation allowed for free circulation of vapor throughout the system. The previously dried disks were placed on the teflon support in an atmosphere of nitrogen or air, and the lid was sealed with high-vacuum grease. A styrofoam cooler insulated the weighing dish against sudden temperature changes and drafts, which tended to cause condensation of water on the disks. The disks were dried a minimum of 48 hr in the P_2O_5 desiccator before applying the lipid film and again were dried for 48 hr. After exposure to tritiated water for a minimum of 25 hr, the disks were broken and placed in scintillation vials for counting. Counting was performed in a Packard Tri-Carb 460C scintillation counter.

RESULTS AND DISCUSSION

The model system. The main goal in developing the new model system was to eliminate oxygen diffusion through the lipid as a variable (14), which would result in the system reaching equilibrium rapidly. Eliminating the dependence of the rate on oxygen diffusion required forming a thin film of lipid, 4–30 μm thick (15), to allow oxygen diffusion to occur rapidly. Other considerations were to maintain a constant oxygen partial pressure and control the RH of the system. Controlling all these factors allowed the study of the effect of individual variables of the lipid autoxidation rate.

Three μl (2.7 mg) of methyl linoleate were used to form the thin film. On the 22 mm glass disk, 2.7 mg of lipid formed a film that was ca. 7.9 μm thick or a vertical layer of at least 10,000 molecules of methyl linoleate. Thus, the lipid was not in a monolayer. The lipid molecules at the silica gel-lipid interface of the disk are oriented largely with the polar heads closely associated with the polar groups of the silica gel, and the hydrophobic tails extending into the bulk lipid of the film (7,8,10). The intermediate bulk lipid, separating the silica gel-lipid and lipid-air interfaces, was free to orient in its lowest free energy form. At the lipid-air interface, the hydrophobic tails of the lipid molecules would orient to the media of the lowest dielectric constant. As the relative humidity was increased in the system, the dielectric constant of the air increased, and the lipid molecules at the air interface probably would orient with the polar heads exposed to the air, where they would bind with water. The amount of water bound to the lipid would increase correspondingly, contributing to the overall effect that humidity has on the rate of oxidation, possibly by binding preferentially to the most polar parts of the lipid molecules and solvating or sterically hindering reaction intermediates.

The thin film had a large surface-to-volume ratio, eliminating the role of oxygen diffusion into the lipid (8), and the small sample size decreased the amount of time needed to reach equilibrium conditions. To assure that oxygen did not become limiting in the reaction system, pure oxygen was used. It has been argued that the use of pure oxygen may affect which termination reaction predominates; however, as long as $pO_2 > 100$ mm Hg, the predominant termination reaction is the homolytic addition of two peroxy radicals (16). Therefore, the system with pure oxygen will have the same oxidation mechanism as a system in which air is used as the source of oxygen and is replaced at regular intervals.

Relative humidity. The necessity of a reservoir of humidity-controlled solution in the model system was clearly demonstrated by the observed increased water-binding during oxidation (Fig. 2). Water bound to the lipid in both air and nitrogen atmospheres, but the oxidizing lipid bound more water than the lipid under nitrogen. Water-hydrogen bonded to the polar oxidation products as they formed. Without the presence of an infinite reservoir of water in the model system, the relative humidity of the system would decrease continually during oxidation of the lipid. The reservoir of saturated solution or water in the reaction vessel replaced the water removed from the atmosphere, preventing any change of RH in the system.

Preliminary studies suggest that the water-binding was primarily at the lipid-air interface because the water/lipid ratio was dependent on lipid layer thickness. This surface phenomenon can be explained by considering the orientation of the hydroperoxides and resulting oxidation products at the lipid-air interface. Water will hydrogen-bond to the polar groups on the oxidation products at the

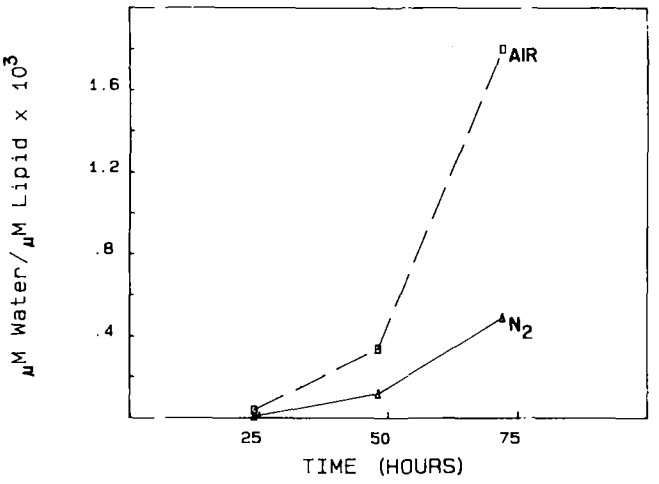

FIG. 2. Micromoles of water bound per μmol methyl linoleate under air and under nitrogen vs time, as determined with tritiated water.

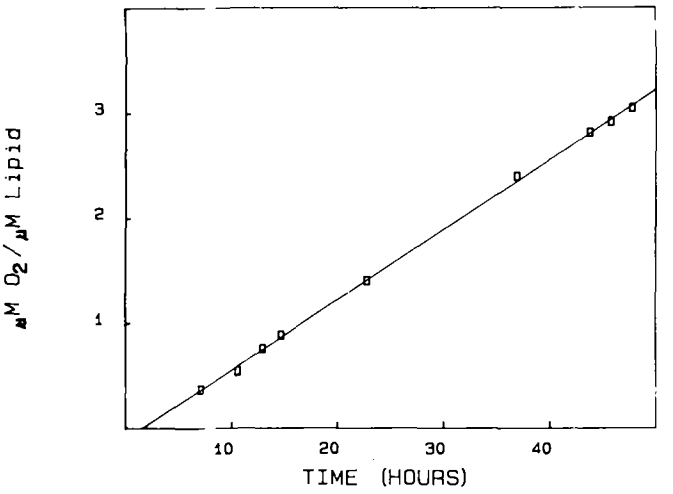

FIG. 3. Typical oxygen uptake for 2.7 mg lipid in a thin film. Oxygen uptake per μmol methyl linoleate vs time, lipid surface-to-volume ratio = 1.27 cm²/μl.

interface, inhibiting them from changing orientation. Thus, most of the lipid-water interactions are expected to occur at the lipid-air interface.

Surface-to-volume ratio, rates and reproducibility. The substrate concentration, oxygen partial pressure and relative humidity of the system were controlled in the model system, and linear uptake of oxygen was observed. The effect of the surface-to-volume ratio of lipid on the rate also was evaluated in the model system. All of the oxygen uptake measurements used to determine the rates reported were performed in the system with a surface-to-volume ratio of 1.27 cm²/μl. Figure 3 shows a typical oxygen uptake vs time plot for a 1.27 cm²/μl thin film. To investigate the effect of the surface-to-volume ratio on the rate of oxidation, a thick film of methyl linoleate with a surface-to-volume ratio of 0.0125 cm²/μl (100-fold smaller than Fig. 3) was placed in the reaction vessel under pure oxygen at 37 C. The nonlinearity of the results shown in Figure 4 demonstrates that the small surface-to-volume ratio sample had not reached steady state at 15 hr, while the large surface-to-volume ratio samples (Fig. 3) took under one hr to reach steady-state.

An analysis of variance was performed at 72% and 100% RH at 37 C by randomly placing the five or six individual slopes in two groups. The beta coefficient (slope) showed no significant difference within runs at the 95% level (Table 1). The alpha regression coefficient (intercept) was not reproducible. However, the degree of oxidation will affect the intercept and result in increasingly greater intercepts as the lipid oxidizes. The intercept would be important if the initiation reactions or the induction period were being studied. Also, the intercept would affect measurement of the induction period. However, neither the initiation reactions nor the induction period were the focus of this study. Once autoxidation is at steady-state, the oxygen uptake rate is independent of the initial condition of the lipid and will remain at steady-state as long as neither oxygen nor substrate become limiting.

The linear rates at all six RH indicate that the reaction was at steady-state; the pseudo-zero order rate generally

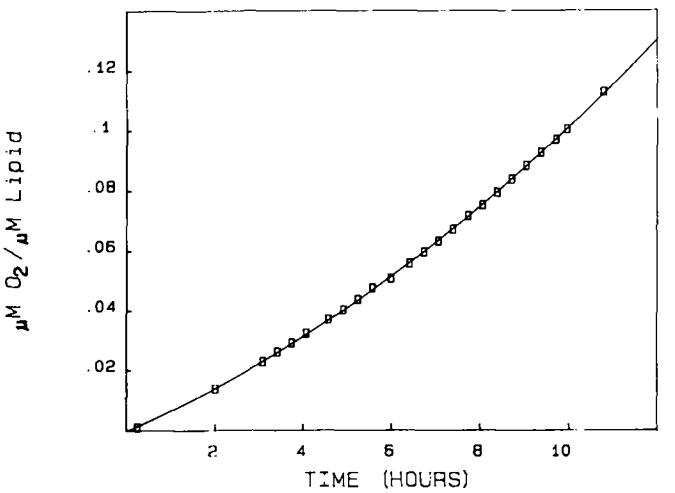

FIG. 4. Oxygen uptake per μmol methyl linoleate vs time for thick film of lipid, surface-to-volume ratio = .0125 cm²/μl.

TABLE 1

Analysis of Variance of Beta Regression Coefficient (Slope) of Lipid Autoxidation Experiments Run at 72 and 100% Relative Humidities in the Model System

Source of variance	df	SS	MS	F
72% RH				
Treatment	1	0.0001348	0.0001348	0.060149
Error	5	0.0112055	0.0022411	
Total	6	0.0113403		
100% RH				
Treatment	1	0.000001	0.000001	0.0007465
Error	4	0.0053584	0.0017861	
Total	5	0.0053594		

TABLE 2

Average Rates of Oxidation and Standard Error of Mean for Methyl Linoleate at Six Relative Humidities

RH (%)	Average N*	Average rate (μM O_2/μM/hr)	Average correlation coefficient
0	13.5	0.0699 ± .0026	0.9958
11	13.5	0.0831 ± .0059	0.9971
32	13.7	0.0915 ± .0064	0.9943
51	11.5	0.0632 ± .0048	0.9928
72	10.7	0.0545 ± .0042	0.9931
100	6.6	0.0388 ± .0035	0.9720

*N, average number of data pairs per linear regression.

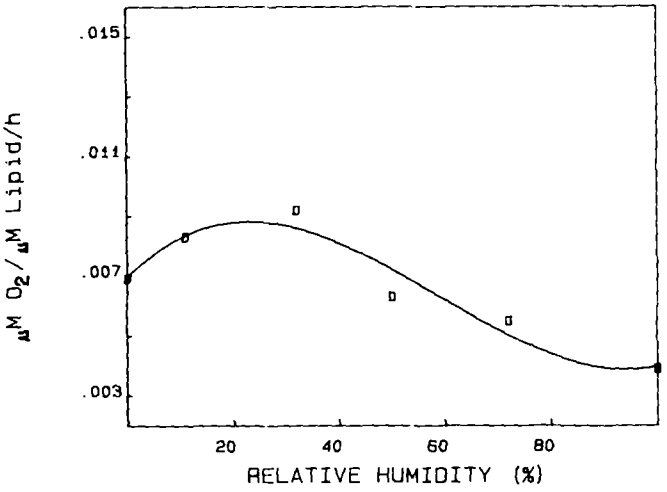

FIG. 5. Rate of oxygen uptake per μmol methyl linoleate per hour vs relative humidity in the model system.

is expected when substrate is not limiting (17-20). Although the sample size was small, gas chromatographic analyses of the oxidized lipid after oxygen uptake measurement consistently showed that the samples were at least 60% unreacted methyl linoleate.

To demonstrate the linearity of the rates, the averages of the correlation coefficients for the linear regressions at six different RH are included in Table 2 with the average rates of oxidation.

Depending on the amount present, water can have a prooxidative or antioxidative effect as demonstrated by the maximum rate at ca. 32% RH and minimum rates at 0% and 100% RH in Figure 5. At 32% RH, the rate reached a maximum, and from 32% to 100% RH the rate decreased with increasing RH. A linear rate of oxidation was observed at all six relative humidities, with no change in the order of the reaction rate with increasing RH and with no change in rate with extent of reaction. The only change in rate of reaction occurred after 60 hr, when substrate became limiting; at that point, the rate of oxygen uptake decreased.

There was no significant induction period, as is common for oxidations of lipid monolayers (10). However, the lipid was not in a monolayer. This would indicate that although the lipid was not in a monolayer, the thin layer of lipid was exposed to an effectively unlimited amount of oxygen, as are monolayers.

To explain the response of the oxidation rate to different RH as observed in the present model system, we propose that the initial addition of water to the oxidizing lipid system increased the rate of oxidation through solvation and stabilization of the propagation transition state, as documented by Lloyd (21). At some relative humidity between 11% and 32%, a maximum stabilization was achieved, resulting in a maximum rate of oxidation. The addition of more water solvated the peroxy radical, decreasing its reactivity through steric hindrance and slowing the overall rate of oxidation.

It is possible that the inhibitory effect on the propagation rate, observed at a solvent polarity beyond the maximum polarity for stabilization, was caused by a decreasing concentration of "reactive" or nonsolvated peroxy radicals. The total concentration of peroxy radicals would not change, but extensive hydrogen-bonding at the higher relative humidities could render some of the radicals nonreactive. Termination possibly may be affected in the same way.

The extent of oxidation measured in the model system was greater than expected and in most cases exceeded one mole of oxygen per mole of lipid. The large amount of oxygen taken up cannot be explained at this time, but we have hypothesized that reactive hydroxyl radicals from hydroperoxide decomposition may abstract hydrogens from already oxidized products, resulting in highly oxidized compounds. In spite of this, it is to be expected that in general the free radical chain reaction will be affected by relative humidity in the same way. Studies are underway to characterize the oxidation products.

ACKNOWLEDGMENTS

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REFERENCES

1. Labuza, T.P. (1971) *CRC Crit. Rev. Food Technol. 2*, 355-405.
2. Krogull, M.K., and Fennema, O. (1987) *J. Agric. Food Chem. 35*, 66-70.
3. Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) *J. Am. Chem. Soc. 103*, 6447-6455.
4. Niki, E., Saito, T., Kawakami, A., and Kamiya, Y. (1984) *J. Biol. Chem. 259*, 4177-4182.
5. Wattenberg, L.V. (1978) *Adv. Cancer Res. 26*, 197-201.
6. McCay, P.B. (1981) *Fed. Proc. 40*, 173-178.
7. Leermakers, P.A., Thomas, H.T., Weis, L.D., and James, F.C. (1966) *J. Am. Chem. Soc. 88*, 5057-5083.
8. Porter, W.L., Levasseur, L.A., Jeffers, J.I., and Henick, A.S. (1971) *Lipids 6*, 16-25.
9. Wu, G.-S., and Mead, J.F. (1977) *Lipids 12*, 965-970.
10. Wu, G.-S., Stein, R.A., and Mead, J.F. (1977) *Lipids 12*, 971-978.
11. Umbreit, W.W., Burris, R.H., and Stauffer, J.F. (1964) *Manometric Techniques*, 4th edn., Burgess Publishing Co., Minneapolis, MN, pp. 46-60.
12. Privett, O.S., and Blank, M.L. (1962) *J. Am. Oil Chem. Soc. 39*, 465-469.
13. Rockland, L.B. (1960) *Anal. Chem. 32*, 1375-1376.
14. Porter, W.L., Levasseur, L.A., and Henick, A.S. (1972) *Lipids 7*, 699-709.

15. Betts, A.T., and Uri, N. (1963) *Nature 199,* 568-569.
16. Porter, N.A., Weber, B.A., Weenan, H., and Khan, J.A. (1980) *J. Am. Chem. Soc. 102,* 5597-5601.
17. Lundberg, W.O., and Chipault, J.R. (1947) *J. Am. Chem. Soc. 69,* 833-836.
18. Taylor, W.F. (1970) *J. Phys. Chem. 74,* 2250-2256.
19. Togashi, H.J., Henick, A.S., and Koch, R.B. (1961) *J. Food Sci. 26,* 186-191.
20. Hyde, S.M., and Verdin, D. (1968) *Trans. Faraday Soc. 64,* 155-162.
21. Lloyd, W.G. (1973) in *Methods in Free-radical Chemistry* (Huyser, E.S., ed.) Vol. 4, Marcel Dekker, Inc., New York, NY, pp. 86-90.

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Preparation of Methyl *cis*-9,*cis*-12,*cis*-15-Octadecatrienoate-15,16-d_2 and Methyl *cis*-9,*cis*-12,*cis*-15-Octadecatrienoate-6,6,7,7-d_4

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Methyl *cis*-9,*cis*-12,*cis*-15-octadecatrienoate-15,16-d_2 was obtained from Wittig coupling of methyl 12-oxo-*cis*-9-dodecenoate, *18*, and 3,4-dideutero-*cis*-3-hexenyltriphenylphosphonium bromide, *16*. Compound *18* was obtained by periodic acid oxidation of methyl 12,13-dihydroxy-*cis*-9-octadecenoate, *17*, obtained from *Vernonia* oil. Compound *18* also was synthesized from methyl oleate as the starting material. The deuterated fragment, *16*, was prepared from 3-hexynol and using Lindlar's catalyst and deuterium gas to introduce the deuterium atoms.

Methyl *cis*-9,*cis*-12,*cis*-15-octadecatrienoate-6,6,7,7-d_4 was prepared by Wittig coupling of 3,6-nonadienyltriphenylphosphonium iodide, *5*, with methyl 9-oxononanoate-6,6,7,7-d_4, *11*. Deuterium atoms were introduced during the synthesis of *11* from 3-butynol and 5-bromopentanoic acid with deuterium gas in the presence of $[Ph_3P]_3$-RhCl. For the preparation of *5*, the 3,6-nonadiynol intermediate was reduced to 3,6-nonadienol with P-2 Nickel and hydrogen.

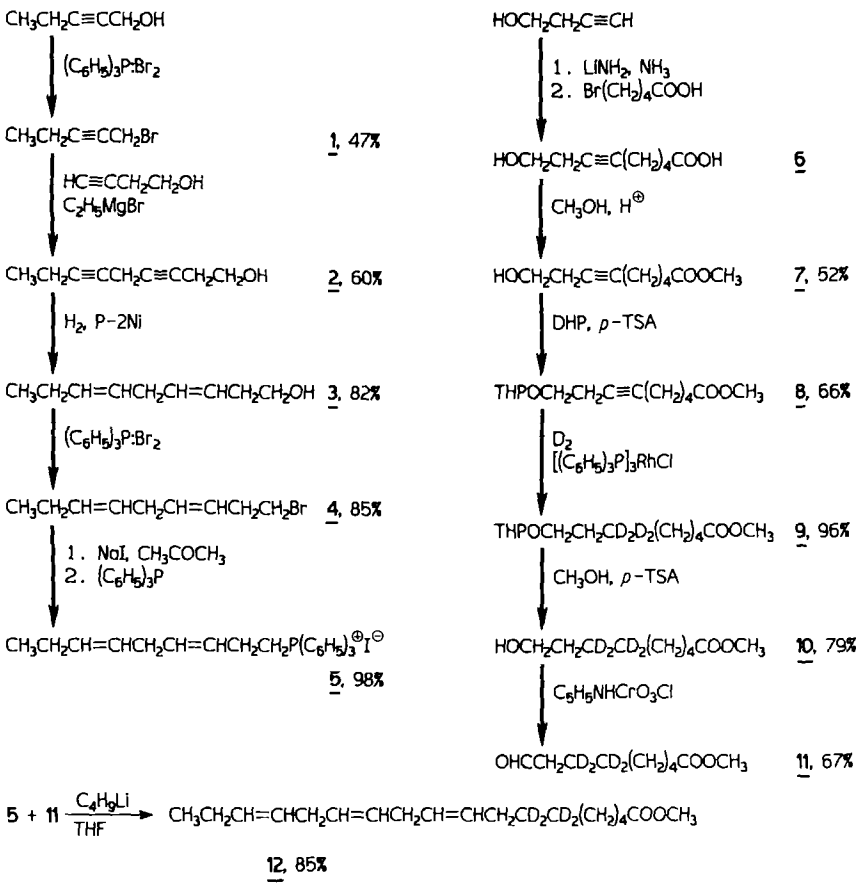
The final products were separated from isomers formed during the synthetic sequences by silver resin chromatography.

Lipids 23, 280–285 (1988).

For our study of the metabolism in humans of fatty acids formed during the hydrogenation of soybean oil (1,2), we have synthesized various deuterated monoenoic (3), dienoic (4) and trienoic acids (5). In all cases, we have been able to avoid putting the deuterium atoms on the double-bonded carbon atoms or on the carbon atoms immediately adjacent to them. As the number of double bonds in the molecule is increased, it becomes more and more difficult to locate the deuterium atoms on carbons that are not joined by a double bond or are immediately adjacent to them. We now have synthesized methyl linolenate containing four deuterium atoms in the 6,6,7,7 positions and methyl linolenate containing two deuterium atoms on the #15 and #16 double-bonded carbon atoms for use in a study to ascertain if there is a "deuterium isotope effect" in the metabolic processes of humans.

DISCUSSION

Methyl linolenate-6,6,7,7-d_4, 12, was obtained by a Wittig coupling of two fragments that were prepared by acetylenic coupling (Scheme 1). For the preparation of 3,6-nonadienyltriphenylphosphonium iodide, 5, 2-pentynol was converted to 1-bromo-2-pentyne, 1, with triphenyl-



SCHEME 1. DHP, Dihydropyran; THP, tetrahydropyranyl; *p*-TSA, *p*-toluene-sulfonic acid; THF, tetrahydrofuran.

phosphine dibromide. Compound 1 then was coupled in a Grignard reaction with 3-butynol to give 3,6-nonadiynol, 2, which was reduced with P-2 Nickel (6) and hydrogen to 3,6-nonadienol, 3. (Lindlar's catalyst is reported [7] to give a mixture of isomeric products with diynes. By contrast, P-2 Nickel reduction goes rapidly to the diene stage in good yield.) Reaction of 3 with triphenylphosphine dibromide gave 1-bromo-3,6-nonadiene, 4, which was converted to the iodide with sodium iodide and to the phosphonium salt, 5, by reaction with triphenylphosphine in acetonitrile.

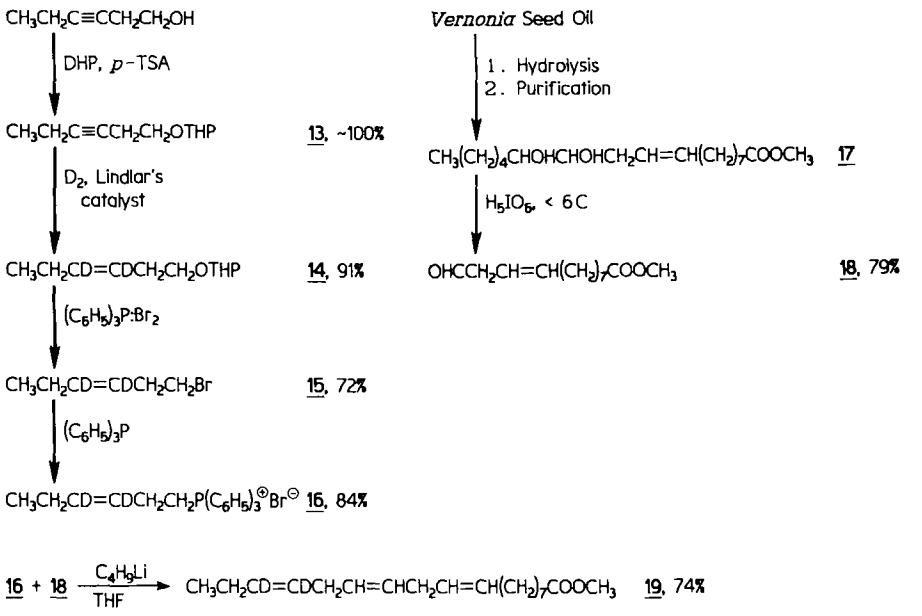
For the aldehyde ester required, methyl 9-oxononanoate-6,6,7,7-d_4, 11, 3-butynol and 5-bromopentanoic acid were coupled by lithium amide in liquid ammonia to give 6, 9-hydroxy-6-nonynoic acid. (The same reaction with methyl 5-bromopentanoate gives a complex mixture of products.) To prevent deuterium-hydrogen exchange during the catalytic deuteration, 6 was esterified to 7 and the hydroxy group was protected as the tetrahydropyranyl ether, 8. Deuteration of 8 with deuterium gas in the presence of *tris*(triphenylphosphine)chlororhodium gave methyl 9-(2-tetrahydropyranyloxy)nonanoate-6,6,7,7-d_4, 9. The tetrahydropyranyl group was removed with methanol and *p*-toluenesulfonic acid to give 10, which was oxidized to 11 with pyridinium chlorochromate (8).

Wittig reaction between 5 and 11 with butyl lithium in tetrahydrofuran gave 12. The all-*cis* isomer was separated from the small amounts of the other isomers generated during the reaction sequence by silver resin chromatography (9).

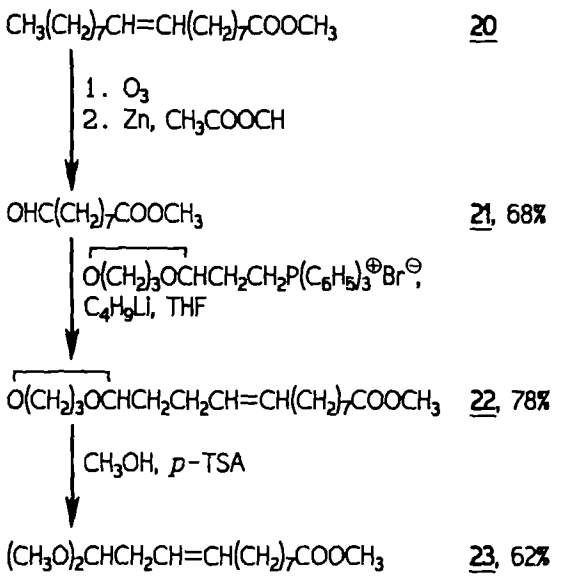
Methyl linolenate-15,16-d_2, 19, was obtained by Wittig coupling (Scheme 2) of 3,4-dideutero-3-hexenyltriphenylphosphonium bromide, 16, and methyl 12-oxo-*cis*-9-dodecenoate, 18, with butyl lithium in tetrahydrofuran. The all-*cis* isomer was separated from the other isomers formed by silver resin chromatography (9). The synthesis of the phosphonium salt started with 3-hexynol, which was converted to the tetrahydropyranyl ether, 13, to prevent hydrogen-deuterium exchange in the subsequent deuteration reaction with deuterium gas and Lindlar's catalyst. The dideuterated tetrahydropyranyl ether, 14, was converted to the bromide, 15, with triphenylphosphine dibromide and to the phosphonium salt, 16, with triphenylphosphine in acetonitrile.

The unsaturated aldehyde ester, 18, was obtained by periodic acid oxidation, below 6 C (10), of methyl *threo*-12,13-dihydroxy-*cis*-9-octadecenoate, 17. This dihydroxy ester was obtained from *Vernonia* oil. The mixture of acids obtained from acetolysis and saponification of *Vernonia* oil contained about 67% dihydroxyoleic acid. This was increased to 84% by distribution between aqueous acetonitrile and petroleum ether and to 96.6% by recrystallization from ethyl ether-petroleum ether.

Compound 18 is a very useful intermediate and was used in our synthesis of mono-, di- and trienoic fatty esters containing a 12,13 double bond (11). Since *Vernonia* oil is not commercially available and was available to us only in limited amounts, we devised a synthesis (Scheme 3) of this material from methyl oleate, which is readily available from the esterification of Palmolyn 100, a commercial source of oleic acid. Palmolyn 100 methyl esters (about 94% methyl oleate), 20, was ozonized, and the ozonide was reduced with zinc and acetic acid to yield 21, methyl 9-oxononanoate (methyl azelaaldehydate). This compound was coupled (12) in a Wittig reaction with [2-(1,3-dioxan-2-yl)ethyl]triphenylphosphonium bromide and butyl lithium in tetrahydrofuran to give the *cis* (75%) and *trans* (25%) isomers of methyl 11-dioxanyl-9-undecenoate, 22. The geometric isomers were separated on a 100% Ag/Na (9) column, and the *cis* isomer was transacetalized with methanol and *p*-toluenesulfonic acid (5) to methyl 12,12-dimethoxy-*cis*-9-dodecenoate, 23. This compound gave the same ^{13}C NMR spectrum as the methyl 12,12-dimethoxy-*cis*-9-dodecenoate obtained by the periodic acid oxidation in methanol of methyl *threo*-12,13-dihydroxy-*cis*-9-octadecenoate obtained from



SCHEME 2. DHP, Dihydropyran; THP, tetrahydropyranyl, *p*-TSA, *p*-toluene-sulfonic acid; THF, tetrahydrofuran.



SCHEME 3. *p*-TSA, *p*-toluenesulfonic acid; THF, tetrahydrofuran.

Vernonia oil (10). Hydrolysis of the acetal ester yields the β, γ-unsaturated aldehyde ester contaminated with small amounts of the α, β-isomer, the quantity of which depends on the reagents and conditions used for the hydrolysis.

If the Wittig reaction between 21 and the dioxanylethylphosphonium salt is carried out under thermodynamic control (4), the reaction mixture will contain a larger percentage of the *trans* isomer of 22. This can be separated and transacetalized to methyl 12,12-dimethoxy-*trans*-9-dodecenoate, which can be used to make other isomers.

EXPERIMENTAL

Reagents. [2-(1,3-Dioxan-2-yl)ethyl]triphenylphosphonium bromide, butyl lithium, triphenylphosphine, dihydropyran, pyridinium chlorochromate, ethyl magnesium bromide, sodium borohydride, 5-bromopentanoic acid, 3-butynol, Molecular Sieve 4A and Lindlar's catalyst (5% Pd on calcium carbonate poisoned with lead) were obtained from Aldrich Chemical Co. (Milwaukee, WI). 2-Pentynol, 3-hexynol and 3-butynol were purchased from Farchan Laboratories (Gainesville, FL). *Tris*(Triphenylphosphine)chlororhodium was obtained from Strem Chemicals (Newburyport, MA) and Palmolyn 100 from Hercules, Inc. (Wilmington, DE).

Procedures. A 50 m × 0.25 mm CPS-2 capillary column (Quadrex Corp., New Haven, CT) was used for analyzing binary mixtures of geometric isomers. For other analyses, a 6 ft × 4 mm column packed with 3% EGSSX on 100/120 GasChrom Q or a 6 ft × 4 mm column packed with 3% OV101 on 80/100 Supelcoport was employed.

^{13}C-NMR. ^{13}C-NMR spectra were recorded with a Bruker WM 300 WB pulsed Fourier transform spectrometer operating at 75.5 MHz. Typically, 2500 transients were collected from solutions in $CDCl_3$, which served as both the internal lock and secondary reference, using 5 mm tubes. Sweep widths of 200 ppm and 8 K real data points limited acquisition time to 0.54 sec and were used to obtain chemical shift values within ±1.85 Hz, i.e. ±0.05 ppm. A pulse width of 3 us (40°) was employed with no delay between pulses. Decoupling power was held to ca. 1 W to provide adequate broadband decoupling power while minimizing sample heating.

Mass spectroscopy. Mass spectra were determined on a Finnigan 4500 mass spectrometer using isobutane chemical ionization with data processing of the isotope distribution against standards (13).

Preparation: 1-bromo-2-pentyne, 1. Triphenylphosphine (164 g, 626 mmol) was dissolved in CH_2Cl_2 (400 ml) in a one-l, three-necked flask equipped with a mechanical stirrer, a low-temperature thermometer, a burette or dropping funnel and a $CaCl_2$ drying tube. While a slow stream of N_2 was passed through the apparatus immersed in an ice bath, bromine (31.8 ml, 99.2 g, 620 mmol) was added dropwise over one hr while the temperature varied between 0 and 6 C. 2-Pentynol (50.27 g, 598 mmol) dissolved in CH_2Cl_2 (50 ml) was added to the slurry over 0.5 hr while the temperature was maintained below 8 C. The ice bath was removed, and about two hr later the reaction mixture was diluted with hexane (400 ml) to precipitate triphenylphosphine oxide. The filtrate and the washings of this precipitate were passed through a column (3 × 50 cm) of silica gel (100 g). Because the bromopentyne codistills with the solvents, the mixture was distilled through a column packed with glass helices (15 mm × 21 cm) to remove the solvent (some bromopentyne still distilled). 1-Bromo-2-pentyne (41.62 g, 47.3% yield) was obtained, bp 130–140 C at atmospheric pressure. ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 12.6; 2, 74.6; 3, 89.4; 4, 13.5; 5, 15.6.

Preparation: 3,6-nonadiynol, 2. 3-Butynol (21.7 g, 310 mmol) was dissolved in tetrahydrofuran (400 ml) in a one-l, three-necked flask equipped with a mechanical stirrer, a low-temperature thermometer, a septum inlet and a condenser and cooled in an ice bath. Ethyl magnesium bromide in tetrahydrofuran (2.04 M, 310 ml, 630 mmol) was added dropwise from a syringe through the septum inlet over one hr while the temperature was maintained between 7 and 14 C. Thirty min later, the ice bath was removed, and one hr after that CuCl (0.5 g) was added. Thirty min later, 1-bromo-2-pentyne (41.17 g, 280 mmol) in tetrahydrofuran (20 ml) was added over 20 min with the temperature maintained at 24–31 C by intermittent use of an ice bath. One hr later, the reaction mixture was heated to the reflux temperature and maintained there for 19 hr. The pale-green opalescent liquid was cooled in an ice bath and acidified with 5 N H_2SO_4 (ca. 110 ml). Water (250 ml) was added, and the mixture was extracted with ethyl ether (1 × 250 ml, 2 × 100 ml). The combined ether layers were washed with saturated NH_4Cl (200 ml) and saturated NaCl (100 ml) solutions and dried over Na_2SO_4. After filtration, the solvent was removed on a rotary evaporator and compound 2 was obtained by distillation, bp 90–97 C @ 1.2 torr (22.64 g, 59.5% yield). ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 60.95; 2, 22.99; 5, 9.60; 7, 81.95; 8, 12.24; 9, 13.74; Unassigned, 76.82, 73.38, 73.05.

Preparation: 3,6-nonadienol, 3. Sodium borohydride (1 g) was dissolved in 95% ethanol (25 ml) and 10% NaOH (1 ml), and the solution was filtered. In a 500 ml round-bottom flask equipped with a magnetic stirrer and connected to an apparatus for maintaining one atmosphere pressure was placed $Ni(C_2H_3O_2)_2.4H_2O$ (4.15 g, 16.6 mmol)

in 95% ethanol (150 ml). The borohydride solution (19 ml, 20 mmol) was added via syringe. There was a rapid evolution of gas, and the mixture turned black. Ethylenediamine (2 ml) was added, and the system was evacuated and flushed several times with H_2. Then, 3,6-nonadiynol (22.64 g, 166 mmol) in 95% ethanol (25 ml) was added, and stirring was started. When absorption of H_2 had almost ceased, the reaction mixture was filtered through a pad of Celite and Darco 6-60 in a Buchner funnel. The pad then was washed with 95% ethanol (2 × 50 ml). The combined ethanol extracts, which had a permanganate color, were concentrated on the rotary evaporator to 65 ml. Water (100 ml) was added, and the mixture was extracted with ethyl ether (3 × 40 ml). The combined ether extracts were washed with saturated NaCl solution (2 × 40 ml) and dried over Na_2SO_4. Distillation gave compound 3 (19.08 g, 81.9% yield), bp 92–98 C @ 4.5 torr. ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 61.95; 2, 30.67; 3, 131.94; 4, 126.77; 5, 25.51; 6, 125.32; 7, 130.93; 8, 20.42; 9, 14.06.

Preparation: 1-bromo-3,6-nonadiene, 4. In a manner similar to the conversion of 2-pentynol to 1-bromo-2-pentyne, 1, 3,6-nonadienol, 3, (19 g, 136 mmol) was treated with triphenylphosphine dibromide to give compound 4 (23.4 g, 85% yield), bp 82–89 C @ 3.75 torr. ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 32.36: 2, 30.83: 3, 131.22; 4, 126.51; 5, 25.69; 6, 126.06; 7, 132.27; 8, 20.57; 9, 14.19.

Preparation: 3,6-nonadienyltriphenylphosphonium iodide, 5. A solution of 4 (23.4 g, 115 mmol) in acetone (50 ml) was added to a solution of NaI (21.6 g, 144 mmol) in acetone (100 ml). The mixture was heated at the reflux temperature for 1.5 hr; the white solid was removed by filtration; and the filtrate was concentrated on the rotary evaporator to an orange liquid and yellow solid (total weight 33.07 g). The liquid was decanted into a 500 ml round bottom flask, and the yellow solid was washed with acetonitrile, which was added to the flask. Triphenylphosphine (35 g, 134 mmol) was added together with the remainder of 200 ml of acetonitrile. Xylene (1 ml) was added as an internal standard, and the reaction mixture was heated at the reflux temperature by means of an oil bath. Samples were analyzed on a 3% OV101 column. After three hr, only 16.6% of the iodo compound remained; after 19 hr, only 1.5% remained. Solvent was removed on the rotary evaporator, the thick liquid was transferred to a beaker and triturated several times with portions of ethyl ether until it deposited crystals. These were washed with ether, pulverized on a clay plate and dried in a vacuum desiccator to give compound 5 (57.8 g, 98% yield). ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 23.22; 2, 20.25; 5, 25.31; 8, 20.14; 9, 13.95; Unassigned: 132.19; 130.68; 125.97; 125.82; 125.77.

Preparation: methyl 9-hydroxy-6-nonynoate, 6 and 7. Liquid ammonia (ca. 800 ml) was charged to a two-l, three-necked flask equipped with a mechanical stirrer and a dry ice-cooled condenser and immersed in a box of Vermiculite insulating material. As catalyst for the formation of lithium amide, $Fe(NO_3)_3.9H_2O$ (0.5 g) was added. Then, lithium metal (9.67 g, 1380 mmol) was added in small pieces over one hr. The reaction mixture became blue-black at first but then changed to a grey slurry. To this was added a solution of 3-butynol (48.3 g, 690 mmol) in tetrahydrofuran (125 ml) over a period of one hr. Two hr later, a solution of 5-bromopentanoic acid (26.4 g, 146 mmol) in tetrahydrofuran (100 ml) was added over 45 min. Stirring was continued for about 1.5 hr. The stirrer was stopped, the Vermiculite was removed, and the ammonia was permitted to evaporate overnight. Ammonia remaining was flushed out with a stream of argon while the reaction vessel was immersed in a warm water bath. Water (60 ml) was added slowly followed by 5 N H_2SO_4 (350 ml) until acidic, and the temperature was maintained at 20 C with an ice bath. The product mixture was extracted into ethyl ether (1 × 250 ml and 2 × 100 ml), and the combined ether extracts were washed with saturated NH_4Cl solution (100 ml) and saturated NaCl solution (100 ml). After drying over Na_2SO_4, the ether was removed on the rotary evaporator to give a reaction mixture (74.66 g). Excess 3-butynol present in this mixture was removed by distillation under reduced pressure, and the material remaining was treated with methanol and H_2SO_4 at the reflux temperature. This reaction mixture was neutralized with solid $NaHCO_3$, concentrated on the rotary evaporator and extracted into ether. Compound 7 (13.75 g, 52% yield) was obtained by distillation, bp 98–115 C @ 0.2 torr. ^{13}C NMR in C_6D_6 (C#, ppm): 1, 173.6; 2, 33.6; 3, 23.5; 4, 28.6; 5, 18.7; 6, 81.4; 7, 77.8; 8, 24.3; 9, 61.7; OCH_3, 51.1.

Preparation: methyl 9-(2-tetrahydropyranyloxy)-6-nonynoate, 8. Methyl 9-hydroxy-6-nonynoate (7 g, 38 mmol) and *p*-toluenesulfonic acid (0.5 g) were dissolved in ethyl ether (10 ml) in a 100 ml round bottom flask equipped with a magnetic stirring bar and a reflux condenser. Dihydropyran (4 g, 48 mmol) in ether (10 ml) was added dropwise. Heat was evolved, and the reaction mixture darkened. After three hr, the reaction mixture was washed with saturated Na_2CO_3 solution (10 ml), dried and distilled under reduced pressure in the presence of a pellet of KOH to give compound 8 (6.74 g, 66% yield) bp 140–147 C @ 0.2 torr. ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 173.5; 2, 33.4; 3, 23.9; 4, 28.2; 5, 18.3; 6, 80.3; 7, 77.2; 8, 20.1; 9, 66.0; OCH_3, 51.2. THP group: 2, 98.5; 3, 30.5; 4, 19.3; 5, 25.4; 6, 61.9.

Preparation: methyl 9-(2-tetrahydropyranyloxy)-nonanoate-6,6,7,7-d_4, 9. Compound 8 (6.7 g, 25 mmol) in benzene (200 ml) was treated with deuterium gas at atmospheric pressure in the presence of *tris*(triphenylphosphine)chlororhodium (1 g) in the manner described previously (3). The benzene was removed on the rotary evaporator, and the residue was passed through a column (1.5 × 25 cm) of silica gel (15 g) with hexane to remove the catalyst. Removal of the solvent gave compound 9 (6.63 g, 96% yield). ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 174.2; 2, 34.1; 3, 24.9; 4–5, 28.9–29.0; 8, 29.4; 9, 67.6; OCH_3, 51.3: THP group: 2, 98.8; 3, 30.8; 4, 19.7; 5, 25.4; 6, 62.3.

Preparation: methyl 9-hydroxynonanoate-6,6,7,7-d_4, 10. Compound 9 (6.63 g, 24 mmol) was stirred in methanol (150 ml) with *p*-toluenesulfonic acid (0.35 g) in a nitrogen atmosphere. Two hr later, solid Na_2CO_3 was added, and the mixture was concentrated to a thick liquid on the rotary evaporator. Saturated Na_2CO_3 solution (40 ml) was added, and the mixture was extracted into ethyl ether (2 × 25 ml) and dried over Na_2SO_4. The drying agent and solvent were removed and compound 10 (3.62 g, 78.7% yield) was obtained by distillation, bp 113–120 C @ 0.35 torr. ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 174.2; 2, 34.0; 3, 24.8; 4–5, 28.8–28.9; 8, 32.4; 9, 62.7; OCH_3, 51.3.

Preparation: methyl 9-oxononanoate-6,6,7,7-d_4, 11. Pyridinium chlorochromate (11.54 g, 53 mmol) was slurried

in CH_2Cl_2 (100 ml, dried over Molecular Sieve 4A) in a 250 ml three-necked flask equipped with a mechanical stirrer, a thermometer and a $CaCl_2$ drying tube. Compound **10** (6.8 g, 35.4 mmol) dissolved in CH_2Cl_2 (20 ml) was added to the orange slurry, which darkened rapidly and deposited a tarry material on the walls of the flask. The temperature was maintained at 20 ± 2 C by intermittent use of an ice bath. Two hr later, ethyl ether (100 ml) was stirred into the mixture and then decanted. This was repeated three times with 25 ml portions of ether. The combined decantates were passed through Florisil (20 g) in a column (1.5 × 25 cm), and the column was flushed with ether (50 ml). The solvents were removed on a rotary evaporator to give a green liquid (6.67 g), that was distilled to give compound **11** (4.47 g, 66.7% yield), bp 96–102 C @ 0.35 torr. ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 174.0; 2, 33.9; 3, 24.8; 4–5, 28.6–28.8; 8, 43.5; 9, 202.6; OCH_3, 51.3.

Preparation: methyl cis-*9*,cis-*12*,cis-*15-octadecatrienoate-6,6,7,7-*d_4, ***12.*** A slurry of **5** (13.5 g, 26.4 mmol) in tetrahydrofuran (100 ml) was prepared in a 250 ml three-necked flask equipped with a mechanical stirrer, a low temperature thermometer, a dropping funnel and a $CaCl_2$ drying tube. A stream of N_2 was passed through the flask and it was cooled in an ice-salt bath to about 1 C. Butyl lithium in hexane (2.42 M, 15 ml, 36 mmol) was added. The temperature rose to about 14 C, and the yellow slurry changed to a deep orange-red color. Compound **11** (4.4 g, 23 mmol) in tetrahydrofuran (12 ml) was added over six min at a temperature of 1–6 C. There was no noticeable color change. The ice bath was removed, and 30 min later the reaction mixture was transferred to a separatory funnel and washed with saturated NaCl solution (100 ml). The organic layer was dried over anhydrous Na_2SO_4. The thick oil (17.33 g) remaining after removal of the solvent was passed through a column (2.2 × 35 cm) of silica gel (40 g) in hexane to give a mixture of isomers (5.87 g, 85% yield). Mass analysis: 0.24% d_2, 0.46% d_3, 98.91% d_4, 0.32% d_5.

To isolate the all *cis* isomer, the reaction mixture (19.03 g) was separated on a silver resin column (9), 5 × 82 cm, Amberlyst XN1010 (40/80 mesh), 100% Ag/H, using as eluant a solution of acetonitrile (300 ml) in methanol (3.7 l). There was obtained 8.6 g (35% overall yield based on the aldehyde ester used) of the all *cis* isomer, better than 98% pure by gas-liquid chromatography on the CPS-2 capillary column. ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 174.2; 2, 34.1; 3, 25.0; 4–5, 28.9–29.1; 8, 27.0; 9, 130.2; 10, 127.7; 11, 25.6; 12, 128.3; 13, 128.3; 14, 25.5; 15, 127.1; 16, 131.9; 17, 20.6; 18, 14.3; OCH_3, 51.4.

Preparation: 2-(3'-hexynyloxy)tetrahydropyran, ***13.*** 3-Hexynol (49 g, 500 mmol) and *p*-toluenesulfonic acid (1.9 g, 10 mmol) were mixed in a 500 ml three-necked flask equipped with a mechanical stirrer, a thermometer and a dropping funnel. While a stream of N_2 was passed through the apparatus, dihydropyran (50.4 g, 600 mmol) was added dropwise over 15 min with the temperature maintained at 23–27 C by intermittent use of an ice bath. The reaction mixture developed a pale pink-amber color. It was passed through a column (3 × 50 cm) of silica gel (100 g) in petroleum ether. Yield of compound **13** was almost 100%.

Preparation: 2-(3'-4'-dideutero-3'hexenyloxy)tetrahydropyran, ***14.*** Compound **13** (45 g, 247 mmol) and ethyl acetate (100 ml) were placed in a 250 ml round bottom flask equipped with a magnetic stirring bar and connected to an apparatus for maintaining one atmosphere pressure. The apparatus was evacuated and flushed three times with D_2. Then, Lindlar's catalyst (1 g) and quinoline (0.8 ml) were added, and a pressure of one atmosphere of D_2 was maintained as the reaction mixture was stirred rapidly. After absorption of D_2 had ceased, the reaction mixture was filtered through a pad of Celite to remove the catalyst. Ethyl acetate was removed by distillation at atmospheric pressure and compound **14** (41.91 g, 91% yield) was obtained by distillation, bp 63–68 C @ 0.4 torr. Mass spectral analysis indicated better than 99% d_2.

Preparation: 1-bromo-3-hexene-3,4-d_2, ***15.*** In a manner similar to the conversion of 2-pentynol to 1-bromo-2-pentyne, **1**, compound **14** was converted to compound **15** in 72% yield, bp 120–140 C. Compound **15** tends to codistill with the solvents employed.

*Preparation: 3,4-dideutero-*cis*-3-hexenyltriphenylphosphonium bromide,* ***16.*** Triphenylphosphine (28.8 g, 110 mmol), compound **15** (17.8 g, 108 mmol) and toluene (1 ml) were mixed in acetonitrile (100 ml) in a 250 ml round bottom flask equipped with a magnetic stirring bar and a reflux condenser. The mixture was heated with stirring at the reflux temperature, and the ratio of bromohexene to toluene was followed by GLC on an OV101 column. After 30 hr, only about 9% of bromohexene remained. Acetonitrile (45 ml) was removed on the rotary evaporator, and the residue was triturated several times with fresh portions of ethyl ether. The white solid that formed was dried in a vacuum desiccator to give compound **16** (39.8 g, 86.4% yield), mp 164–167. ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 23.07; 2, 20.15; 5, 20.03; 6, 13.7; aromatic ring: 1, 118.03; 2, 133.50; 3, 130.37; 4, 134.95.

*Preparation: methyl 12-oxo-*cis*-9-dodecenoate,* ***18.*** Methyl 12,13-dihydroxy-*cis*-9-octadecenoate (50 g, 152 mmol) was dissolved in tetrahydrofuran (150 ml) in a 500 ml Erlenmeyer flask equipped with a magnetic stirring bar and a low-temperature thermometer and immersed in an ice-salt bath. Paraperiodic acid, H_5IO_6 (43.5 g, 190 mmol) was dissolved in H_2O (87 ml) and cooled in an ice bath. The periodic acid solution was added in portions over 40 min so that the temperature did not rise above 4 C. The reaction mixture was diluted with H_2O (150 ml), the organic layer was separated, and the aqueous layer was extracted with hexane (2 × 50 ml). The combined organic layers were washed with H_2O (1 × 25 ml) and dried over Na_2SO_4 and Drierite. After removal of the drying agents and solvent, the residue was freed of any other volatiles (especially hexanal) by use of a vacuum pump at room temperature. The residue was then distilled through a falling film molecular still (0.05 torr, refluxing xylene) to give compound **18** (27.36 g, 79% yield).

Preparation: methyl cis-*9*,cis-*12*,cis-*15-octadecatrienoate-15,16-*d_2, ***19.*** Compound **16** (39.03 g, 91.4 mmol) was slurried in tetrahydrofuran (200 ml) in a 500 ml three-necked flask equipped with a mechanical stirrer, a low-temperature thermometer, a dropping funnel and a $CaCl_2$ drying tube. A stream of N_2 was maintained through the apparatus as it was cooled in an ice-salt bath. Butyl lithium in hexane (2.5 M, 40 ml, 100 mmol) was added in portions with the temperature varying between 0 and 18 C. Then compound **18** (27.25 g, 120 mmol) in tetrahydrofuran (10 ml) was added over 12 min to the blood

red reaction mixture. The maximum temperature was 7 C, and the reaction mixture formed a pale-tan slurry. The ice bath was removed, and about one hr later the reaction mixture was shaken with saturated NaCl solution (100 ml). The organic layer was dried over Na_2SO_4 and Drierite. After filtration, the solvent was removed on the rotary evaporator, and the residue (71.71 g) was placed on a column (3 × 50 cm) containing silica gel (100 g) in hexane. Elution with hexane yielded a mixture of isomers (19.81 g, 73.7% yield). This mixture was separated on a 5 × 82 cm column packed with Amberlyst XN1010 (40/80 mesh) 100% Ag/H resin (9) with the eluant being acetonitrile (300 ml) in methanol (3.7 l), to give the all-*cis* isomer (7.69 g, 28.4% yield based on the phosphonium salt used). Mass spectral analysis: 0.41% d_0, 0.29% d_1, 99.26% d_2. ^{13}C NMR in $CDCl_3$ (C#, ppm): 1, 174.1; 2, 34.0; 3, 24.8; 4-7, 29.0-29.4; 8, 27.1; 9, 130.1; 10, 127.6; 11, 25.3-25.5; 12, 128.1; 13, 128.1; 14, 25.3-25.5; 17, 20.3; 18, 14.1.

Preparation: methyl 9-oxononanoate, 21. Compound 21 was prepared in 64% yield essentially as previously described (14) but with the use of Sudan Red (0.01 g in 10 g of cyclohexane) as an indicator of the completion of the ozonolysis and Palmolyn 100 methyl esters as the source of methyl oleate.

Preparation: methyl 11-dioxanyl-9-undecenoates, 22. Compound 22, bp 140-152 C @ 0.2 torr, was obtained in 78% yield by the method previously described (5,12) for the synthesis of another dioxanyl ester. Mass spectroscopy showed a parent peak at 284 m/z and a large peak at 87 m/z corresponding to the dioxanyl group. ^{13}C NMR chemical shifts were consistent with the structure assigned. The *cis* and *trans* isomers of 22 (73.4% *cis*) were separated on a 3.5 × 30 cm column of 100% Ag/Na (9) Amberlyst XN1010 ion exchange resin (200-270 mesh). From a sample of 1.84 g, there was obtained 1.19 g (88% of theory) of the *cis* isomer.

Preparation: methyl 12,12-dimethoxy-cis-9-dodecenoate, 23. Compound 23, bp 132-140 C @ 0.25 torr, was obtained in 62% yield from 22 by a method described (5,12). The ^{13}C NMR spectrum is identical to that of the dimethylacetal ester prepared by periodic acid oxidation in methanol (10) of methyl 12,13-dihydroxy-*cis*-9-octadecenoate. During GC/MS analysis, the dimethyl acetal apparently loses CH_3OH to give methyl 12-methoxy-9,11-dodecadienoate, which gives a parent peak at 240 m/z and an M-31 peak at 209 m/z. The base peak is at 97 m/z corresponding to $CH_3\overset{\oplus}{O}=CH_2\text{-}(CH=CH)_2$.

Purification of 12,13-dihydroxy-cis-9-octadecenoic acid. (1) By distribution between aqueous acetonitrile and petroleum ether. The mixture of acids obtained from the acetolysis and saponification of *Vernonia anthelmintica* seed oil (15) was analyzed by GLC on 3% EGSSX after the acid group had been converted to the methyl ester and the hydroxy groups had been converted to a dioxolane with acetone and $BF_3.CH_3OH$. This acid mixture (301 g) showed 67% dihydroxyoleic acid. It was suspended in a mixture of acetonitrile (700 ml) and H_2O (70 ml) and extracted with petroleum ether (3 × 250 ml). Removal of the CH_3CN-H_2O gave a product (204.3 g) that analysis showed to contain 84% dihydroxyoleic acid. (2) By crystallization from ethyl ether-petroleum ether. A sample (230 g) of *Vernonia* acids containing 84% dihydroxyoleic acid was dissolved with stirring in ethyl ether (400 ml). Addition of petroleum ether (500 ml) caused a haziness that was cleared up by addition of ethyl ether (20 ml). The mixture was cooled in an ice bath for several hours with stirring, and the precipitate that formed was filtered with suction and washed with petroleum ether to remove a small amount of contaminating dark oil. The solid was pulverized and air-dried to give a product (61.26 g) analysis of which showed 96.6% dihydroxyoleic acid, 1.57% linoleic acid, 0.38% oleic acid, 0.36% palmitic acid and 1.04% of a material eluting after dihydroxyoleic acid. Mp 49-50 C.

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REFERENCES

1. Emken, E.A. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.), pp. 99-129, American Oil Chemists' Society, Champaign, IL.
2. Emken, E.A., Dutton, H.J., Rohwedder, W.K., and Adlof, R.O. (1980) *Lipids 15,* 864-871.
3. Rakoff, H., and Emken, E.A. (1978) *J. Labelled Compd. Radiopharm. 15,* 233-252.
4. Rakoff, H., and Emkem, E.A. (1982) *J. Labelled Compd. Radiopharm. 19,* 19-33.
5. Rakoff, H. (1984) *Chem. Phys. Lipids 35,* 117-125.
6. Brown, C.A., and Ahuja, V.K. (1973) *J. Chem. Soc. Chem. Commun.,* 553-554.
7. Kajiwara, T., Sekiya, J., Odake, Y., and Hatanaka, A. (1977) *Agric. Biol. Chem. 41,* 1481-1484.
8. Corey, E.J., and Suggs, J.W. (1975) *Tetrahedon Lett.,* 2647-2650.
9. Adlof, R.O., and Emken, E.A. (1981) *J. Am. Oil Chem. Soc. 58,* 99-101.
10. Rakoff, H., Adlof, R.O., and Emken, E.A. (1979) *Synth. Commun. 9,* 185-199.
11. Rakoff, H., and Emken, E.A. (1983) *J. Am. Oil Chem. Soc. 60,* 546-552.
12. Stowell, J.C., and Keith, D.R. (1979) *Synthesis,* 132-133.
13. Rohwedder, W.K. (1985) *Prog. Lipid Res. 24,* 1-18.
14. Rakoff, H. (1982) *Prog. Lipid Res. 21,* 225-254.
15. Rakoff, H., and Emken, E.A. (1978) *J. Am. Oil Chem. Soc. 55,* 564-566.

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Uptake of Fatty Acids by the Developing Rat Brain

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Polyunsaturated fatty acids are avidly taken up by the developing rat brain. To explore the specificity of this process, [1-^{14}C]labeled 16:0, 18:2n-6, 18:3n-3, and 22:6n-3 each were co-injected with [^{3}H]18:1n-9 into the jugular vein of two-wk-old functionally hepatectomized and sham-operated control rats. The radioactivities present in the brain, liver and serum were assessed 30 min after injection. Uptake of labeled fatty acids into brain lipids steadily increased with increasing degree of unsaturation, with more than twice as much uptake of 22:6n-3 compared to 16:0. Phosphatidylcholine was the principal radioactive species in the brain except for animals injected with [1-^{14}C]22:6n-3, in which more of the label was incorporated into phosphatidylethanolamine. Determination of water-soluble oxidation products in the brain and serum revealed that the greater uptake of the more unsatrated fatty acids did not result from differences in rates of degradation.
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The n-3 fatty acids are highly enriched in nervous tissue and are assumed to have important functions. Nutritional studies have suggested that these species are essential in the diet and that the symptoms produced by n-3 fatty acid deficiency are distinct from those produced by n-6 fatty acid deficiency (1–4). While the pathway leading to the synthesis of docosahexaenoic acid (22:6n-3), the principal n-3 fatty acid in the brain, is well-understood (5), the process by which this fatty acid actually reaches the brain is less clear. It is known that in rats, most of the adult complement of brain 22:6n-3 is laid down after birth during the period of brain development preceding myelination (6,7). However, it is not known to what extent the brain synthesizes 22:6n-3 from precursor molecules such as 18:3n-3, or how specifically or in what quantity the brain takes up 22:6n-3 and other fatty acids from the circulation. A number of studies have examined the question of uptake of unsaturated fatty acids by the developing brain (8–13), with delivery of the isotope either orally or by intraperitoneal injection. We decided to compare brain uptake of 22:6n-3, 18:3n-3 and other fatty acids more directly by injecting the labeled fatty acids intravenously and by removing the liver from the circulation to reduce secondary metabolism of the label.

MATERIALS AND METHODS

All isotopes were purchased from NEN Research Products (Boston, MA) and [9,10-^{3}H]18:1 n-9 was purified by silica gel thin layer chromatography (TLC) before use. In each experiment, ca. 5 μCi of ^{14}C fatty acid was used together with 5 μCi of [^{3}H]18:1 to provide an internal reference. To prepare samples for injection, labeled fatty acids (in ethanol solution) were dried under nitrogen, redissolved in 0.6 ml 20% rat serum in normal saline (14), and stirred magnetically for 15 min. Before injection, an aliquot was removed for determination of radioactivity.

The animals used were unfasted two-wk-old male Sprague-Dawley rat pups (median age 15 days, range 13–16 days) weighing 37.2 ± 3.6 g. At this age, the uptake of polyunsaturated fatty acids by the brain is maximal (6,7). Dams were fed standard laboratory chow ad libitum. The rat pups were anesthetized with 0.03 ml i.p. of a ketamine cocktail (100 mg/ml ketamine, 20 mg/ml xylazine, and 10 mg/ml acepromazine) and were functionally hepatectomized by placing a Biemer clip (Aesculap Instruments Corp., Burlingame, CA) on the porta hepatis to interrupt circulation to the liver. The livers of these animals immediately turned pale and remained so except for occasional periods when they again became pink during "deep breaths." This probably explains the presence of radioactivity in these livers (Table 1). Sham-operated controls were treated in an identical manner except that the liver was not compromised.

Fatty acids were injected into the jugular vein by means of an indwelling catheter consisting of a snapped-off 23-gauge needle connected to a three-way stopcock by PE-50 tubing. Patency was verified by drawing back a small amount of blood before injection. Approximately 0.55 ml of the fatty acid solution was slowly introduced and was followed by a wash of 0.2 ml 20% rat serum in normal saline.

After 30 min, a small amount of blood was drawn from the vena cava and the animal decapitated. The brain and liver (weighing ca. 1 g each) then were quickly removed, rinsed in saline, blotted and frozen. These tissues were extracted according to Folch et al. (15) and an aliquot from both the aqueous and organic phases counted in Beckman HP-b cocktail on a Beckman LS-9000 scintillation counter capable of simulaneously determining ^{3}H and ^{14}C. The ^{3}H in the aqueous phase was not quantitated, since labeled oxidation products arising from [9,10-^{3}H]18:1 cannot be compared with those arising from [1-^{14}C] labeled fatty acids.

The ^{14}C and ^{3}H DPM in the organic phase, expressed per gram wet weight tissue, were dose-corrected to 5 μCi. These individual values then were normalized to compare uptake of the various [^{14}C] labeled fatty acids relative to the internal standard, [^{3}H]18:1. The normalization was carried out by multiplying each individual [^{14}C] uptake by the ratio (mean uptake of [^{3}H]18:1 from all experiments):(respective individual uptake of [^{3}H]18:1). The means of the normalized uptakes of the various ^{14}C labeled fatty acids were then compared by multiple t-tests, using the BonFerroni inequality (16) to control the overall α-level. No attempt was made to quantify the radioactivity remaining in the carcass or exhaled as CO_2.

To examine the distribution of label within the tissues, the total lipids of brain, liver and serum were fractionated by silica gel TLC with hexane/chloroform/ethyl ether/acetic acid (80:10:10:1.5). Brain lipids also were

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Abbreviations: TLC, thin layer chromatography; VLDL, very low density lipoprotein.

TABLE 1

Uptake of IV-administered Fatty Acids by the Liver and Developing Brain of 15-day-old Rats*

	[1-^{14}C]-labeled fatty acid				[9,10-^{3}H]18:1n-9 (Reference)
	16:0	18:2n-6	18:3n-3	22:6n-3	
	DPM/g × 10^4				
Brain					
Hepatect.	4.3 ± 0.2^{a}	5.6 ± 0.1^{b}	7.4 ± 0.7^{c}	11.1 ± 1.2^{d}	3.6 ± 0.6
Sham**	3.3 ± 0.2^{a}	3.1 ± 0.4^{a}	4.2 ± 0.3^{b}	6.3 ± 2.3^{a,b}	2.1 ± 0.7
Liver					
Hepatect.	20 ± 3	19 ± 1	17 ± 2	21 ± 2	20 ± 18
Sham	126 ± 25^{a}	148 ± 10^{a}	102 ± 32^{a}	408 ± 111^{b}	122 ± 66

*Mean ± S.D. (n = 3, except n = 12 for 18:1) of the lipid-soluble tissue radioactivity 30 min after co-injection of 5 μCi of ^{14}C-labeled fatty acid and 5 μCi [^{3}H]18:1n-9 into the jugular vein. Uptake of ^{14}C in each animal was normalized to the mean uptake of [^{3}H]18:1 as described in Methods. Values with unlike superscripts within a given row are different from each other at a BonFerroni $P < 0.04$.

**Sham-operated vs hepatectomized different at BonFerroni $P < 0.05$.

chromatographed on silica gel 60 plates with methyl acetate/propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, v/v/v/v/v) (17) to separate phospholipid classes. Scintillation cocktail was sprayed onto the plates to visualize the bands, which then were scraped directly into counting vials. Because free fatty acid was the major component of the serum lipids but only a minor component of the brain lipids of hepatectomized animals and because of the seven-fold higher ^{3}H:^{14}C ratio in the free fatty acid fractions of the serum lipids vs brain lipids (data not shown), it was possible to determine by successive approximations that the amount of ^{14}C in the brain lipids that actually was due to contaminating serum was very low, on the order of 1-2%.

The fatty acid composition of the brain, liver and serum of two-wk old rat pups was determined by capillary gas chromatography as described (1).

RESULTS

The IV injection of radiolabeled fatty acids into two-wk-old rat pups resulted in a measurable accumulation of radioactivity in the developing brain of these animals 30 min after their injection (Table 1). Substantially more radioactivity reached the brain of the functionally hepatectomized pups. There was a steady and significant increase in uptake of [1-^{14}C] fatty acid as the degree of unsaturation increased; 22:6n-3 showed more than twice as much uptake as 16:0 and considerably more than 18:2n-6.

Most of the label in the brain was present as phospholipid, with small amounts of labeled cholesterol, fatty acid and triglyceride (Table 2). For 16:0, 18:1, 18:2n-6 and 18:3n-3, the bulk of the phospholipid radioactivity was found in the phosphatidylcholine fraction. In contrast, 22:6n-3 was incorporated principally into phosphatidylethanolamine. Interestingly, there was roughly twice as much label in the brain phosphatidylinositol fraction of the animals injected with 18:3n-3 than in the phosphatidylinositol of the animals injected with the other fatty acids. There was little change in the distribution of radioactivity in the brains of sham-operated controls except that slightly less material was present in phospholipid, with a corresponding increase in the amount of cholesterol and triglyceride (data not shown).

The presence of a portion of the label from each experiment in the cholesterol fraction of the brain lipids indicates that some fatty acid must have been oxidized to acetate with subsequent synthesis of cholesterol. This reuse of breakdown products by the developing rat brain has been noted before (11,12,21,29,30). About twice as much cholesterol was formed from 18:3n-3 than from the other ^{14}C-labeled fatty acids, suggesting that more of this fatty acid was oxidized in the brain. This observation is supported by the data in Table 3, in which water-soluble ^{14}C was measured in the brain, liver and serum of both the hepatectomized and control pups. Twice as much degradation product(s) was present in the brains of animals injected with 18:3n-3 than in animals injected with the other fatty acids. The special susceptibility of 18:3n-3 to oxidation has been noted (12,13,18).

It also can be seen in Table 3 that there was no decrease in oxidation products with increasing degree of unsaturation of the injected fatty acid. In fact, the opposite was true, i.e. there was somewhat more oxidation of those fatty acids that accumulated to a greater extent in the brain. This also was the case in serum. In general, there was about a three-fold increase in the amount of oxidation product measured in the brains of sham-operated controls than in hepatectomized animals.

The distribution of serum lipids was also investigated by TLC (Table 4). When the liver was removed from the circulation, the bulk of the serum lipid radioactivity (representing about 1% of the injected dose) still was present as fatty acid 30 min after injection, although significant quantities of 18:3n-3 and 22:6n-3 were found as phospholipid. However, in sham-operated controls roughly 3% of the injected dose was found in serum lipids, and most of this was present as triglyceride. This probably represents very low density lipoprotein (VLDL) secreted by the liver. The anomalously high proportion of ^{3}H label found in the fatty acid fraction of serum lipids

TABLE 2

Distribution of Radioactivity in Brain Lipids 30 Min After IV Injection of Labeled Fatty Acids*

	[1-^{14}C]-labeled fatty acid				[9,10-^{3}H]18:1n-9 (Reference)
	16:0	18:2n-6	18:3n-3	22:6n-3	
	Percent total DPM				
PtdCho**	54.2 ± 4.2^{a}	52.3 ± 0.1^{a}	35.6 ± 1.4^{b}	26.0 ± 0.8^{c}	45.3 ± 2.1
PtdSer	3.1 ± 0.6	4.8 ± 1.1	5.9 ± 1.3	4.7 ± 1.9	4.4 ± 0.9
PtdIns	3.7 ± 0.9^{a}	7.0 ± 0.6^{b,c}	11.7 ± 3.0^{b}	5.1 ± 1.2^{a,c}	5.2 ± 0.9
PtdEtn	12.5 ± 2.6^{a}	14.4 ± 1.2^{a,b}	19.0 ± 2.4^{b}	42.2 ± 1.1^{c}	22.1 ± 2.7
Total phospholipid	73.5 ± 1.6	78.5 ± 0.5	72.3 ± 0.8	78.0 ± 1.3	77.1 ± 1.8
Cholesterol	6.9 ± 0.8^{a}	5.8 ± 0.1^{a}	10.5 ± 1.4^{b}	4.0 ± 0.3^{c}	5.3 ± 1.7
Fatty acid	6.3 ± 2.5	8.8 ± 0.4	5.5 ± 0.3	4.2 ± 2.5	10.1 ± 2.8
Triglyceride	9.6 ± 0.7	4.5 ± 0.4	6.3 ± 0.2	8.0 ± 2.3	5.0 ± 0.7

*Lipid extracts from the brains of the hepatectomized animals shown in Table 1 were separated by one-dimensional TLC, with the data summarized as means ± SD (n = 3, except n = 12 for 18:1). Values with unlike superscripts within a given lipid fraction are different from each other at a BonFerroni $P < 0.05$.

**Phosphatidylcholine.

TABLE 3

Water-soluble Oxidation Products of [1-^{14}C]-labeled Fatty Acids*

	[1-^{14}C]-labeled fatty acid			
	16:0	18:2n-6	18:3n-3	22:6n-3
	DPM/g × 10^{3}			
Hepatectomized rats				
Brain	2.7 ± 0.1^{a}	3.4 ± 0.5^{a,c}	7.0 ± 0.6^{b}	3.9 ± 0.3^{c}
Liver	5.1 ± 3.3	2.9 ± 2.3	6.1 ± 4.4	3.8 ± 1.0
Serum**	6.1 ± 3.6^{a}	11.4 ± 1.1^{a}	47.6 ± 3.6^{b,c}	53.8 ± 38.8^{a,c}
Sham-operated rats				
Brain	12.4 ± 1.7^{a}	9.7 ± 1.0^{a,c}	20.7 ± 3.6^{b}	5.3 ± 2.5^{c}
Liver	20.0 ± 2.1^{a}	18.2 ± 2.0^{a}	39.1 ± 2.8^{b}	17.5 ± 14.3^{a,b}
Serum**	14.4 ± 12.0^{a}	16.2 ± 3.3^{a}	92.5 ± 31.8^{b}	43.5 ± 26.4^{a,b}

*Mean ± SD (n = 3) of the ^{14}C radioactivity remaining in the aqueous phase after Folch extraction of the brain, liver and serum of the animals shown in Table 1. Values within each row with unlike superscripts are different from each other at a Bonferroni $P < 0.05$.

**Serum values are DPM/ml × 10^{3}.

TABLE 4

Distribution of Radioactivity in Serum Lipids after IV Injection of Labeled Fatty Acids*

	[1-^{14}C]-labeled fatty acid				[9,10-^{3}H]18:1n-9 (Reference)
	16:0	18:2n-6	18:3n-3	22:6n-3	
	Percent total DPM				
Hepatectomized rats					
Phospholipid	8.5 ± 4.4^{a}	15.2 ± 10.4^{a,b}	38.7 ± 10.8^{b}	27.5 ± 14.1^{a,b}	4.4 ± 1.6
Cholesterol	29.7 ± 7.7^{a}	5.3 ± 2.1^{b}	2.2 ± 0.3^{b}	8.9 ± 4.6^{b}	3.7 ± 1.1
Fatty acid (free)	51.4 ± 6.0^{a}	76.9 ± 12.9^{b}	49.3 ± 11.2^{a,b}	62.3 ± 14.6^{a,b}	90.1 ± 2.5
Triglyceride	5.5 ± 7.0	1.3 ± 1.7	1.9 ± 2.5	0.4 ± 0.2	1.2 ± 1.4
Sham-operated rats					
Phospholipid	5.2 ± 1.2^{a}	5.0 ± 1.4^{a}	13.8 ± 2.5^{b}	15.9 ± 13.1^{a,b}	4.8 ± 1.5
Cholesterol	3.0 ± 0.1	4.2 ± 0.6	4.6 ± 1.9	5.3 ± 2.5	4.0 ± 0.9
Fatty acid (free)	8.0 ± 0.7	9.5 ± 0.5	11.6 ± 6.2	13.4 ± 4.3	42.8 ± 14.7
Triglyceride	79.2 ± 1.4^{a}	78.5 ± 2.2^{a,b}	66.3 ± 6.6^{b}	64.5 ± 16.5^{a,b}	45.6 ± 15.3

*Serum lipids from the animals in Table 1 were separated by silica gel TLC, with the data summarized as means ± SD (n = 3, except n = 12 for 18:1). Values with unlike superscripts within a given lipid fraction are different at a BonFerroni $P < 0.05$.

from both the hepatectomized and control rats is an artifact due to degradation products which co-migrate with fatty acid, since this anomaly was not seen when [1-^{14}C]18:1n-9 was injected (data not shown).

Despite interruption of the normal blood supply to the liver in the functionally hepatectomized animals, some radioactivity was found in these livers (Table 1). However, there was no differential metabolism of the various fatty acids. On the other hand, sham-operated controls showed considerable uptake of fatty acid by the liver, roughly 30–40 times that of the brain on a per gram basis.

The liver showed an even stronger preference for 22:6n-3 than the brain, with the liver accumulating four times more radioactivity from this fatty acid than from the other fatty acids, which were not significantly different in their uptakes. This selectivity by the liver for 22:6n-3 has been noted (10). The distribution of label in liver lipids was quite different from brain. For 18:2n-6, the label was found about equally in phospholipid and triglyceride. Two-thirds of the label from 18:3n-3 was present in triglyceride and about one-third in phospholipid, while the reverse was true for 22:6n-3 (data not shown). Very little of the radioactivity was present in cholesterol or cholesteryl ester.

To compare the tissue uptake of the injected fatty acids with the relative amounts of these fatty acids present in the tissues, the fatty acid composition of brain, liver and serum from two-wk-old rat pups was determined (Table 5). The relative uptake of [1-^{14}C] labeled 16:0, 18:2n-6, 18:3n-3 and 22:6n-3 by the brain and liver bore no relationship to the relative concentration of each species in the total fatty acids of these tissues. With regard to the high uptake of 22:6n-3 by the two-wk-old liver, the concentration of 22:6n-3 in liver total fatty acids was twice as high as in normal adult rat liver (19).

TABLE 5

Fatty Acid Composition of Brain, Liver and Serum of Two-Wk-Old Rats

	% Total fatty acids[a]		
	Brain (n = 5)	Liver (n = 5)	Serum (n = 5)
14:0	0.8 ± 0.3	1.0 ± 0.2	2.3 ± 0.6
16:0	27.3 ± 0.6	23.8 ± 1.1	23.5 ± 1.6
18:0	19.3 ± 1.4	15.3 ± 0.5	10.2 ± 0.2
Total sat.	49.1 ± 0.7	40.9 ± 1.5	36.6 ± 2.2
16:1n-7	1.5 ± 0.2	0.6 ± 0.1	1.2 ± 0.1
18:1n-9	15.4 ± 0.5	8.9 ± 0.8	9.9 ± 0.7
20:1n-9	0.5 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
Total mono.	17.6 ± 0.3	9.8 ± 0.8	11.5 ± 0.9
18:2n-6	1.3 ± 0.3	13.6 ± 0.7	20.7 ± 0.3
20:3n-6	0.6 ± 0.0	0.8 ± 0.1	0.8 ± 0.1
20:4n-6	13.1 ± 0.7	15.9 ± 0.8	19.4 ± 2.0
22:4n-6	2.9 ± 0.1	0.7 ± 0.1	0.3 ± 0.1
22:5n-6	1.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
Total n-6	19.8 ± 0.5	31.8 ± 0.6	42.1 ± 1.9
18:3n-3	N.D.	0.3 ± 0.1	0.3 ± 0.1
20:5n-3	0.06 ± 0.02	0.8 ± 0.1	1.6 ± 0.1
22:5n-3	0.4 ± 0.0	3.3 ± 0.2	1.7 ± 0.1
22:6n-3	12.5 ± 0.8	12.8 ± 0.8	5.7 ± 0.8
Total n-3	13.0 ± 0.8	17.2 ± 0.8	9.4 ± 0.9

[a]Means ± S.D.
N.D., not detected.

DISCUSSION

During the suckling period, the developing rat brain accumulates most of its eventual adult complement of polyunsaturated fatty acids, including 22:6n-3. This process occurs at a linear and maximal rate 10–16 days after birth (6,7). For this reason, we chose 15-day-old suckling rats for our study. We compared the brain uptake of IV-injected, ^{14}C labeled 16:0, 18:2n-6, 18:3n-3 and 22:6n-3 by relating each of these to the uptake of a co-injected reference fatty acid, [^{3}H]18:1n-9. Brain uptake in the functionally hepatectomized animals increased steadily with increasing degree of unsaturation, suggesting that the brain is not neutral in its selection of fatty acids from the circulation. Functional hepatectomy improved the uptake of fatty acid into the developing brain to a lesser extent than it did in adult rats injected with 16:0 or 18:2n-6 (23,26).

The difference in uptake between 18:3n-3 and 22:6n-3 was unexpectedly small. For example, Sinclair (11) found that oral feeding of 18:3n-3 and 22:6n-3 to two-wk-old rats led to the accumulation in the brain of 10 times more label from 22:6n-3 than from 18:3n-3. A similar disparity was noted by Hassam and Crawford (9) for orally fed 18:2n-6 vs 20:4n-6. Apparently, when the potential for metabolism and/or oxidation of the delivered fatty acid is reduced by bypassing the intestines and removing the liver from the circulation, a more moderate selectivity by the brain for the more unsaturated fatty acids is revealed. One might argue that the relative accumulation of label seen in Table 1 is the result of less degradation of the more unsaturated fatty acids, either before they entered the brain, or after. This is not consistent with the data in Table 3, however, where it is seen that more not fewer oxidation products of 18:3n-3 and 22:6n-3 were detected in the brain and serum of functionally hepatectomized rats.

Phosphatidylcholine was the most highly labeled brain lipid after IV injection of radioactive 16:0, 18:1n-9, 18:2n-6 and 18:3n-3, a result consistent with previous experiments on adult and suckling rats (12,13,20–23). However, the presence in phosphatidylethanolamine of the major portion of the label from IV-injected [1-^{14}C]22:6n-3 was not expected based on the results from intracranial injection (24). Fifteen min after direct injection of 22:6n-3 into the brain of 18-day-old rats, roughly 50% of the label was in phosphatidylcholine and about 40% in phosphatidylethanolamine. The distribution was approximately unchanged after 60 min. This contrasts with only 26% of the label in phosphatidylcholine and 42% in phosphatidylethanolamine after 30 min in our experiments. Thus, it is possible that 22:6n-3 passing to the brain through the capillary endothelium enters a different compartment than 22:6n-3 injected directly into the brain.

A question not addressed by our study was the degree to which the injected fatty acids were desaturated and chain-elongated. One possible explanation for the relatively high uptake of label from [1-^{14}C]18:3n-3 would entail the metabolism of this compound to 22:6n-3 followed by enhanced uptake by the brain relative to 18:3n-3 proper. However, the striking difference in the fate of the

label from [1-^{14}C]18:3n-3 and [1-^{14}C]22:6n-3 suggests that 18:3n-3 did not enter the brain as "22:6n-3." With the present data, we cannot make any statement about the extent to which each fatty acid was further elaborated after reaching the brain.

There is controversy over the chemical form of the essential fatty acids taken up by the brain. That is, it is not clear whether free fatty acid (bound to albumin or perhaps α-fetoprotein in the case of 22:6n-3 [25]) is the preferred form of lipid taken up by the brain, as has been proposed (26). For example, most of the 22:6n-3 in the serum of two-wk-old suckling rats actually is present in phosphatidylcholine, and most of the 18:3n-3 is present as triacylglycerol (23). It also is not clear to what extent the liver is involved. The presence of significant quantities of 22:6n-3 in the serum lipoproteins of rat pups suckling mothers fed diets high in 18:3n-3 has been interpreted as implying a role for the liver in the desaturation/elongation of essential fatty acids, and their repackaging for export to the developing brain (27). On the other hand, Cook (28) found the developing brain itself to have a high capacity for desaturation of essential fatty acids. By studying the kinetics of lipid uptake after IV injection of 18:3n-3, 22:6n-3 and other fatty acids in a variety of chemical forms and vehicles, we should be able to better characterize the uptake process and the role of the liver.

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REFERENCES

1. Neuringer, M., Connor, W.E., Lin, D.S., Barstad, L., and Luck, S. (1986) *Proc. Natl. Acad. Sci. USA 83,* 4021-4025.
2. Neuringer, M., and Connor, W.E. (1986) *Nutr. Rev. 44,* 285-294.
3. Tinoco, J. (1982) *Prog. Lipid Res. 21,* 1-45.
4. Neuringer, M., Connor, W.E., VanPetten, C., and Barstad, L. (1984) *J. Clin. Invest. 73,* 272-276.
5. Naughton, J.M. (1981) *Int. J. Biochem. 13,* 21-32.
6. Kishimoto, Y., Davies, W.E., and Radin, N.S. (1965) *J. Lipid Res. 6,* 532-536.
7. Sinclair, A.J., and Crawford, M.A. (1972) *J. Neurochem. 19,* 1753-1758.
8. Hassam, A.G., Sinclair, A.J., and Crawford, M.A. (1975) *Lipids 10,* 417-420.
9. Hassam, A.G., and Crawford, M.A. (1976) *J. Neurochem. 27,* 967-968.
10. Sinclair, A.J., and Crawford, M.A. (1972) *FEBS Lett. 26,* 127-129.
11. Sinclair, A.J. (1975) *Lipids 10,* 175-184.
12. Dhopeshwarkar, G.A., and Subramanian, C. (1975) *Lipids 10,* 238-241.
13. Dhopeshwarkar, G.A., and Subramanian, C. (1975) *Lipids 10,* 242-247.
14. Babcock, R., Medwadowski, P., Miljanich, J., Tinoco, J., and Lyman, R.L. (1976) *Proc. Soc. Exp. Biol. Med. 152,* 298-301.
15. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
16. Miller, R.G. (1966) *Simultaneous Statistical Inference,* p. 8, McGraw-Hill, New York, NY.
17. Vitiello, F., and Zanetta, J.P. (1978) *J. Chromatogr. 166,* 637-640.
18. Leyton, J., Drury, P.J., and Crawford, M.A. (1987) *Br. J. Nutr. 57,* 383-393.
19. Tinoco, J., Williams, M.A., Hincenbergs, I., and Lyman, R.L. (1971) *J. Nutr. 101,* 937-946.
20. Illingworth, D.R., and Glover, J. (1973) *J. Neurochem. 21,* 1403-1414.
21. Dhopeshwarkar, G.A., Subramanian, C., and Mead, J.F. (1973) *Biochim. Biophys. Acta 296,* 257-264.
22. Dhopeshwarkar, G.A., and Subramanian, C. (1976) *J. Neurochem. 26,* 1175-1179.
23. Sinclair, A.J. (1975) *Proc. Nutr. Soc. 34,* 287-291.
24. Onuma, Y., Masuzawa, Y., and Waku, K. (1984) *Biochim. Biophys. Acta 793,* 80-85.
25. Uriel, J., Naval, J., and Laborda, J. (1987) *J. Biol. Chem. 262,* 3579-3585.
26. Dhopeshwarkar, G.A., and Mead, J.F. (1973) *Adv. Lipid Res. 11,* 109-142.
27. Nouvelot, A., Delbart, C., and Bourre, J.M. (1986) *Ann. Nutr. Metab. 30,* 316-323.
28. Cook, H.W. (1978) *J. Neurochem. 30,* 1327-1334.
29. Gozlan-Devillierre, N., Baumann, N.A., and Bourre, J.M. (1976) *Biochimie 58,* 1129-1133.
30. Gozlan-Devillierre, N., Baumann, N.A., and Bourre, J.M. (1976) *Biochim. Biophys. Acta 528,* 490-496.

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Mevalonate 5-Pyrophosphate Decarboxylase in Isolated Villus and Crypt Cells of Chick Intestine

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Mevalonate 5-pyrophosphate decarboxylase was studied in isolated enterocytes obtained from duodenal, jejunal and ileal villi and crypts. In our assay conditions, decarboxylase activity was linear for 60 min and up to 0.3 mg of protein. The subcellular location of decarboxylase in chick enterocytes was investigated. About 94% of the total activity was recovered in the cytosol. The distribution of enzyme activity in epithelial cells also was studied. Maximal specific activity was found in cell fractions from jejunum followed by ileum and duodenum. About 80% of total activity was recovered in the villus cells, indicating an active role of these cells in cholesterogenesis. Ileal cells showed the highest cholesterol content. In all the intestinal epithelial cells assayed, free cholesterol represented about 95% of the total cholesterol.
Lipids 23, 291-294 (1988).

The small intestine plays a key role in cholesterol metabolism both because it is the site for absorption of dietary sterol and because it is one of the major sites for de novo cholesterogenesis (1). However, current understanding of the regulation of cholesterol metabolism in the small intestine is incomplete. From the early work of Dietschy and Siperstein (2), it was postulated that cholesterol synthesis in the intestine, unlike the liver, was not inhibited by dietary cholesterol. Likewise, Shefer et al. (3) observed no differences in intestinal 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity in rats fed 2% cholesterol when compared with that of rats fed normal chow, while cholesterol feeding resulted in the inhibition of intestinal reductase in hamsters (4) and guinea pigs (5,6). Recently, we have reported the inhibitory effect of cholesterol feeding on chick intestine HMG-CoA reductase (7).

Although HMG-CoA reductase is considered to be the rate-limiting enzyme for cholesterogenesis in liver and other tissues (8,9), the existence of secondary sites of regulation beyond mevalonic acid (MVA) formation has been suggested (10,11). In this sense, we have shown that feeding a 2% cholesterol diet from hatching resulted in a significant inhibition of mevalonate 5-pyrophosphate (MVAPP) decarboxylase from chick intestine, while mevalonate kinase and mevalonate 5-phosphate (MVAP) kinase remained unaltered (12). These results, together with the developmental pattern shown by MVAPP decarboxylase during embryonic (13) and postnatal (14) periods as well as its changes in response to different nutritional conditions (12,15-17), demonstrate the important role of this enzyme in the regulation of cholesterol biosynthesis.

Until now, the enzymes responsible for the intestinal transformation of mevalonate to isopentenyl pyrophosphate have not been characterized. The purpose of this study was to investigate the distribution of MVAPP decarboxylase in the epithelial cells isolated from chick duodenum, jejunum and ileum in a villus-to-crypt gradient to compare with that of HMG-CoA reductase and to resolve the controversy over which intestinal cell type (villus or crypt) is responsible for cholesterol synthesis.

MATERIALS AND METHODS

White Leghorn male chicks *(Gallus domesticus)* 30-35 days old were used. Newborn animals were obtained from a commercial hatchery and maintained fed ad libitum on a commercial diet (Sanders A-00) in a chamber with a light cycle from 09.00 to 21.00 hr and controlled temperature. D-[2-^{14}C]Mevalonate 5-pyrophosphate (ammonium salt) and [6-^{3}H]thymidine were supplied by Amersham International (Amersham, U.K.). All other reagents used were analytical grade.

Chicks were killed by decapitation at the same hour every day (2 p.m.). Intestines were removed, chilled and then divided into three segments designated duodenum (between gizzard and biliar conduct junction), jejunum (a 12-cm region from 15 cm after biliar conduct junction) and ileum (a 12-cm region proximal to the ileo-cecal junction). Enriched isolated epithelial cell fractions from different locations along the villus-crypt axis were prepared according to Weiser (18) with the modification suggested by Raul et al. (19), consisting in the eversion of the intestine fragment prior to incubation in the chelation buffers. To define the enterocyte fraction isolated after various time intervals, alkaline phosphatase was determined as marker enzyme for differentiated villus cells (18), while thymidine kinase, determined according to Klemperer and Haynes (20) and Breitman (21) was regarded as an indicator for crypt cells. Acid phosphatase, measured by the method of Bingham and Zittle (22), was characteristic for both cell types (23).

Incubations carried out according to Weiser (18) for 20-min intervals up to a total of 160 min yielded succesive cell fractions with varying activities of the marker enzymes. Thus, the specific activity of alkaline phosphatase decreased gradually in cells progressively harvested, while the specific activity of thymidine kinase increased in the final cell fraction relative to the initial fraction. Acid phosphatase showed a practically similar specific activity in all fractions recovered, so that the observed differences in alkaline phosphatase and thymidine kinase were not due to differences in cell stability.

According to these results, the various cell fractions were combined in four separate pools designated as upper (0-40 min), mid (40-80 min) and lower (80-120 min) villus cells and as crypt cells (120-160 min) from duodenum and jejunum. In ileum, similar pools were considered according to the following intervals of time: 0-30 min, 30-60 min, 60-90 min and 90-120 min, respectively. These terms represented only a functional classification based upon the enzyme content of these groups of enterocytes and probably should not be taken as a literal indication of the anatomic sources for the various cells

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Abbreviatons: MVA, mevalonic acid; MVAP, mevalonate 5-phosphate; MVAPP, mevalonate 5-pyrophosphate.

(24). Protein contents were determined by the method of Lowry et al. (25) using bovine albumin as standard.

Subcellular fractionation was carried out by centrifugation of homogenates obtained by sonication of cell suspensions. The homogenates were centrifuged at 500 × g for 15 min to collect the nuclear fraction. The supernatant fraction was centrifuged at 15,000 × g for 15 min to yield mitochondria. Microsomal and cytosolic fractions were isolated from the postmitochondrial supernatant by centrifugation at 105,000 × g for 60 min. The entire fractionation was carried out at 0–4 C.

MVAPP decarboxylase activity was assayed using [2-^{14}C]MVAPP as substrate of the reaction as previously described (14). Enzyme activity was expressed as nmol of alcohols liberated/min/mg protein.

Lipids were extracted with chloroform/methanol (2:1, v/v) according to Folch et al. (26). Total and free cholesterol contents were determined by enzymatic colorimetric methods using "Test-Combination Cholesterol" or "Test-Combination Free Cholesterol," respectively, from Boehringer Mannheim GmbH (Germany).

RESULTS

The first group of experiments was undertaken to define the optimal conditions for measuring MVAPP decarboxylase activity in a large pool of isolated enterocytes obtained from both duodenal, jejunal and ileal villi and cripts. As shown in Figure 1, decarboxylase activity was linear with respect to the amount of cell protein in the incubation vial up to 0.3 mg. Likewise, enzyme activity was studied as a function of incubation time. As can be seen in Figure 2, the specific activity was linear up to 60 min of incubation.

The subcellular localization of MVAPP decarboxylase in chick villus and crypt cells is reported in Table 1. Maximal specific activity was found in the cytosolic fraction, with a recovery of about 94% of total activity.

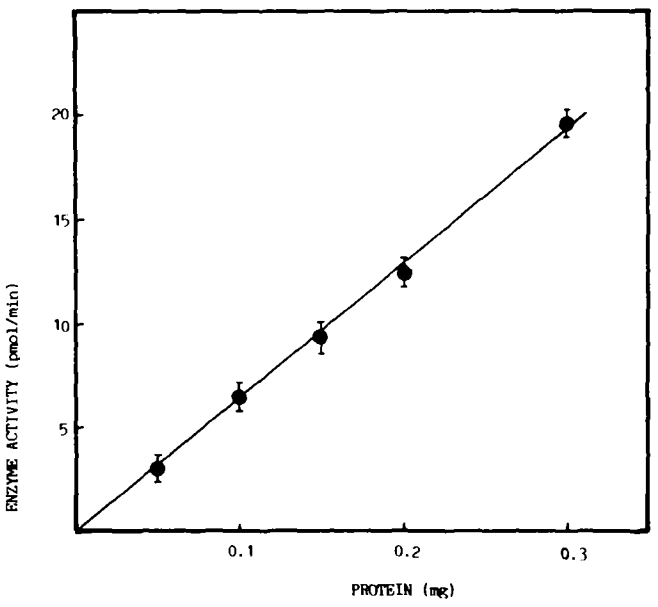

FIG. 1. Effect of protein content of a large pool of enterocytes isolated from duodenal, jejunal and ileal villi and crypts on MVAPP decarboxylase activity. Results are expressed as means ± SEM of three determinations.

The distribution of MVAPP decarboxylase activity in epithelial cells isolated from duodenum, jejunum and ileum, as described in Materials and Methods, is shown in Table 2. Maximal specific activity was found in all fractions from jejunum, although differences with duodenum and ileum were small. The crypt and lower villus cells showed the highest specific activities in each intestinal fragment. When multiplied by the mass of protein recovered in each of these fractions, more than 80% of total decarboxylase activity was found in villus cells. Duodenum showed the highest total activity of the three fragments assayed.

The total cholesterol content was determined in chick villus and crypt cells. As can be seen in Table 3, ileum showed the highest cholesterol content, followed by duodenum and jejunum. In all the intestinal epithelial cells assayed, free cholesterol represented about 95% of total cholesterol.

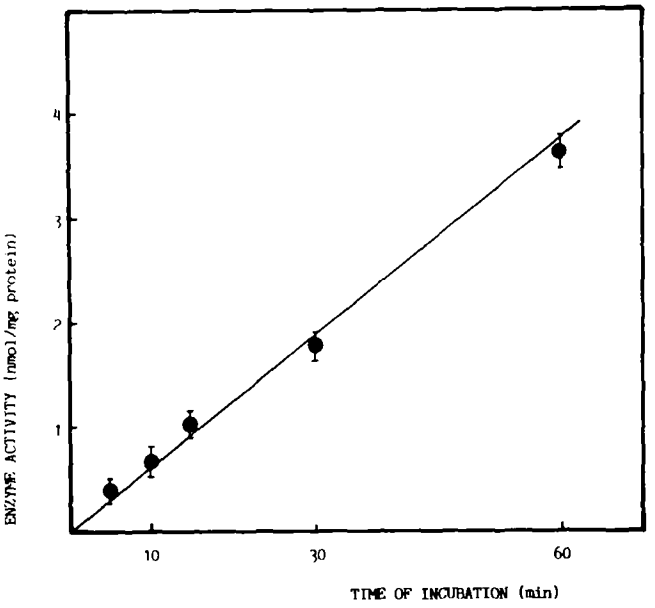

FIG. 2. Effect of incubation time on MVAPP decarboxylase activity from chick enterocytes. Results are expressed as means ± SEM of three determinations with a large pool of enterocytes isolated from duodenal, jejunal and ileal villi and crypts.

TABLE 1

Subcellular Distribution of MVAPP Decarboxylase in Chick Enterocytes[a]

Fraction	Specific activity (pmol/min/mg protein)	% total activity
Homogenate	111.6 ± 2.1	100
Nuclei	23.0 ± 1.5	4
Mitochondria	12.4 ± 1.5	1
Microsomes	7.6 ± 1.1	1
Cytosol	423.0 ± 6.0	94

[a]Results are expressed as means ± SEM of three determinations carried out using a large pool of isolated enterocytes obtained from duodenal, jejunal and ileal villi and crypts.

TABLE 2

MVAPP Decarboxylase Activity in Villus and Crypt Cell Fractions from Chick Duodenum, Jejunum and Ileum[a]

	Duodenum			Jejunum			Ileum		
Cell fraction	Sp. act.	Total act.	% Total act.	Sp. act.	Total act.	% Total act.	Sp. act.	Total act.	% Total act.
Upper villus	30.4 ± 0.1	2.7 ± 0.1	39	32.5 ± 4.8	0.9 ± 0.1	28	30.0 ± 1.5	0.6 ± 0.1	17
Mid villus	29.4 ± 2.3	1.7 ± 0.1	24	44.7 ± 1.5	1.1 ± 0.1	34	34.0 ± 1.7	1.2 ± 0.1	34
Lower villus	37.5 ± 1.8	1.3 ± 0.1	18	52.7 ± 1.1	0.8 ± 0.1	25	43.4 ± 1.6	1.2 ± 0.1	34
Crypt	41.4 ± 5.0	1.4 ± 0.2	19	46.8 ± 0.1	0.4 ± 0.1	13	52.6 ± 7.0	0.5 ± 0.1	15

[a]Results are expressed as mean values ± SEM of the specific activities (pmol/min/mg protein) or total activities (nmol/min) obtained in three determinations as well as percentages found in each fraction of total activity in duodenum, jejunum and ileum, respectively.

TABLE 3

Cholesterol Content of Epithelial Cells from Chick Duodenum, Jejunum and Ileum[a]

	Duodenum		Jejunum		Ileum	
Cell fraction	Total	Free	Total	Free	Total	Free
Upper villus	9.2 ± 0.3	9.0 ± 0.3	8.7 ± 0.1	8.3 ± 0.7	16.0 ± 0.2	15.6 ± 0.2
Mid villus	10.9 ± 0.3	9.9 ± 0.1	8.7 ± 0.1	8.2 ± 0.1	16.6 ± 0.2	16.4 ± 0.5
Lower villus	11.1 ± 0.4	9.7 ± 0.9	8.3 ± 0.2	7.7 ± 0.2	14.9 ± 0.3	14.1 ± 0.3
Crypt	9.1 ± 0.2	8.7 ± 0.1	12.4 ± 0.5	12.3 ± 0.5	17.1 ± 0.3	16.0 ± 0.3

[a]Results are expressed as mean values ± SEM of total and free cholesterol content (μg/mg protein) obtained in three determinations in chick duodenum, jejunum and ileum.

DISCUSSION

In this work, we have studied for the first time the distribution of MVAPP decarboxylase in isolated villus and crypt cells. The subcellular fraction with the highest specific activity was the cytosol, in agreement with previous results obtained on this (12) and other chick mevalonate-activating enzymes (28). However, it is important to remark that HMG-CoA reductase activity isolated from epithelial cells from chick intestine was found in the microsomal and mitochondrial fractions (unpublished results), thus demonstrating the existence of a gross contamination of the different organelle fractions similar to that reported in rat intestine (29,30). Because of this, we have used in this work the whole homogenate for comparative studies with reductase.

The distribution of MVAPP decarboxylase in the different intestinal segments was practically similar to that found for HMG-CoA reductase; jejunal cells showed the maximal specific activities of both enzymes. About 80% of total decarboxylase activity was recovered in the villus cells, thus indicating an active role of these cells in cholesterol synthesis. Similar results previously were obtained on the distribution of HMG-CoA reductase: about 70% of its total activity was found in the upper and the mid-villus fraction (unpublished results).

Concerning the cholesterol content of intestinal epithelial cells, the fact that absorption of luminal sterols in the chick occurs mainly in the proximal portion (31) led us to postulate that a large part of the cholesterol content of the ileum may be synthesized in situ. This intestinal segment was the richest in total cholesterol, sterol that accumulated almost exclusively in the free form. High free cholesterol levels would be in good agreement with the low acyl-CoA:cholesterol acyltransferase specific activity previously reported for this region (32). Likewise, the accumulation of free cholesterol did not determine an inhibition of cholesterogenic enzyme activities HMG-CoA reductase and MVAPP decarboxylase, as has been reported in other physiological conditions in which the variations observed in free cholesterol were parallel to those found in both enzyme activities (13,32).

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REFERENCES

1. Stange, E.F., Suckling, K.E., and Dietschy, J.M. (1983) *J. Biol. Chem. 258*, 12868-12875.
2. Dietschy, J.M., and Siperstein, M.D. (1967) *J. Lipid Res. 8*, 97-104.
3. Shefer, S., Hauser, S. Lapar, V., and Mosbach, E.H. (1973) *J. Lipid Res. 14*, 400-405.
4. Ho, K.J. (1975) *Proc. Soc. Exp. Biol. Med. 150*, 271-277.
5. Swann, A., Wiley, M.H., and Siperstein, M.D. (1975) *J. Lipid Res. 16*, 360-366.
6. Turley, S.D., and West, C.E. (1976) *Lipids 11*, 571-577.
7. Alejandre, M.J., Ramirez, H., Segovia, J.L., and Garcia-Peregrin, E. (1985) *Ann. Nutr. Metab. 29*, 111-118.
8. Siperstein, M.D. (1970) *Curr. Top. Cell. Reg. 2*, 65-100.
9. Schroepfer, G.J. Jr. (1981) *Annu. Rev. Biochem. 50*, 585-621.

10. Ramachandran, C.K., and Shah, S.N. (1976) *Biochem. Biophys. Res. Commun. 69,* 42-47.
11. Jabalquinto, A.M., and Cardemil, E. (1981) *Arch. Biochem. Biophys. 210,* 132-139.
12. Gonzalez-Pacanowska, D., Marco, C., Garcia-Martinez, J., and Garcia-Peregrin, E. (1985) *Biochim. Biophys. Acta 833,* 449-455.
13. Marco, C., Gonzalez-Pacanowska, D., Linares, A., and Garcia-Peregrin, E. (1983) *Neurochem. Res. 8,* 711-721.
14. Gonzalez-Pacanowska, D., Marco, C., Garcia-Martinez, J., and Garcia-Peregrin, E. (1984) *Int. J. Biochem. 16,* 845-847.
15. Gonzalez-Pacanowska, D., Marco, C., Garcia-Martinez, J., Linares, A., and Garcia-Peregrin, E. (1985) *Nutr. Rep. Int. 31,* 121-127.
16. Gonzalez-Pacanowska, D., Marco, C., Garcia-Martinez, J., and Garcia-Peregrin, E. (1986) *Biochim. Biophys. Acta 875,* 605-609.
17. Marco, C., Gonzalez-Pacanowska, D., Segovia, J.L., and Garcia-Peregrin, E. (1986) *Biochim. Biophys. Acta 878,* 238-242.
18. Weiser, M.M. (1973) *J. Biol. Chem. 248,* 2536-2541.
19. Raul, F., Simon, P., Kedinger, M., and Haffen, K. (1977) *Cell Tissue Res. 176,* 167-178.
20. Klemperer, H.G., and Haynes, G.R. (1968) *Biochem. J. 108,* 541-546.
21. Breitman, T.R. (1963) *Biochim. Biophys. Acta 67,* 153-155.
22. Binghan, E.W., and Zittle, C.A. (1963) *Arch. Biochem. Biophys. 101,* 471-477.
23. Fortin-Magana, R., Hurwitz, R., Herbst, J.J., and Kretchner, N. (1970) *Science 167,* 1627-1628.
24. Stange, E.F., and Dietschy, J.M. (1983) *J. Lipid Res. 24,* 72-82.
25. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
26. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
27. Marco, C., Morillas, L.G., and Garcia-Peregrin, E. (1986) *Biochim. Biophys. Acta 875,* 599-604.
28. Garcia-Martinez, J., Segovia, J.L., Suarez, M.D., and Garcia-Peregrin, E. (1976) *Biochem. Biophys. Res. Commun. 72,* 202-208.
29. Hubscher, G., West, G.R., and Brindley, D.N. (1965) *Biochem. J. 97,* 929-642.
30. Field, F.J., Erickson, S.K., Shrewsbury, M.A., and Cooper, A.D. (1982) *J. Lipid Res. 23,* 105-113.
31. Sklan, D., Budowsky, P., Ascarelli, I., and Hurwitz, S. (1973) *J. Nutr. 103,* 1299-1305.
32. Marco, C., Alejandre, M.J., Gonzalez-Pacanowska, D., Segovia, J.L., and Garcia-Peregrin, E. (1985) *Int. J. Biochem. 17,* 271-274.

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Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: X. Volatile Thermal Decomposition Products of Methyl Linolenate Dimers

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High-molecular weight compounds previously were found to be important secondary products from autoxidation of polyunsaturated fatty esters. The contribution of dimers to oxidative deterioration was investigated by analyzing their volatile thermal decomposition products by capillary gas chromatography-mass spectrometry. Dimers were isolated by gel permeation chromatography from autoxidized linolenate and from the corresponding monohydroperoxides, cyclic peroxides and dihydroperoxides. Major volatile decomposition products identified from these oxidative dimers were similar to those formed from the corresponding monomeric hydroperoxides. However, dimers from linolenate hydroperoxides produced more propanal and methyl 9-oxononanoate than the corresponding monomers but less methyl octanoate and much less or no 2,4-heptadienal and 2,4,7-decatrienal. Significant differences in minor volatile products also were observed between dimeric and monomeric products of methyl linolenate oxidation compounds. Mechanisms are suggested for the formation of volatile decomposition products from different dimeric structures. These dimers are believed to be important sources of volatile compounds contributing to flavor and oxidative deterioration of fats.
Lipids 23, 295–298 (1988).

Much work has been reported on the monomeric and volatile oxidation products of fats, but relatively little information is available on the nature of the high-molecular weight products, especially those from linolenate. Early reports indicated that high-molecular weight compounds from linoleate and linolenate may be important sources of flavor compounds in vegetable oils (1–6). The thermal polymerization of oxidized methyl linolenate in the absence of air generated a complex mixture of oxygen-containing compounds that included 70% dimers (4).

Dimer structures with conjugated diene systems were reported from linoleate hydroperoxide oxidized at 38 C in the presence of a free radical initiator and absence of oxygen (7). More recently, peroxidic dimers that contained either allylic hydroperoxide or hydroxy substituents were identified in linoleate autoxidized at 30 C (8–11). Small amounts of ether-linked dimers also were identified from decomposed linoleate hydroperoxides (11). In the decomposition of linoleate dimers at 30 C, a mixture of hydroxy aldehydes, hydroxy esters and hydroxy aldehyde esters were identified (12).

We recently identified the dimers formed either directly during the autoxidation of methyl linolenate or by decomposing linolenate hydroperoxides, hydroperoxy epidioxides and dihydroperoxides at 40 C. The linolenate dimers carbon linkages (13). Different volatile decomposition products were identified from methyl linolenate hydroperoxides when decomposed either thermally at 150 C or catalytically with ferric chloride and ascorbic acid at room temperature (14). This paper extends these studies to clarify the decomposition pathways of dimeric products isolated from oxidized linolenate, its hydroperoxides and corresponding secondary oxidation products.

EXPERIMENTAL

Procedures for the preparation of oxidation products from methyl linolenate were as described (15). Autoxidized methyl linolenate, pure linolenate mono- and di-hydroperoxides, and hydroperoxy epidioxides were decomposed with air at 40 C. The decomposition mixtures were dissolved in methylene chloride and separated by gel permeation chromatography into oligomeric, monomeric and low-molecular weight fractions (13,14). The oligomeric fractions consisted mainly of dimeric components with molecular weights varying from 625–930 (13).

Volatile thermal decomposition products of monomeric and dimeric fractions formed after injection at 200 C in a gas chromatograph (GC) (Perkin-Elmer, model Sigma 300, Norwalk, CT), were analyzed with a capillary column (DB 1701, J & W Scientific Co., Rancho Cordova, CA) cooled to −20 C with liquid nitrogen. Column temperature was programmed to 260 at 5 C per min with a final hold of 10 min. Integrated relative peak areas obtained with flame ionization detection were calculated as weight percentage of methyl hexanoate used as internal standard (14). Volatile decomposition products separated and characterized by capillary gas chromatography-mass spectrometry (GC-MS), were identified by matching their mass spectra and by comparing their GC retention data to those of known reference compounds (16,17).

RESULTS

Dimeric fractions isolated by gel permeation chromatography from methyl linolenate autoxidized at 40 C varied from 0.1 to 10.1% by weight in direct proportion to the peroxide values (13). Dimers were isolated by gel permeation chromatography in 60–90% yields from pure monohydroperoxides, 65% from hydroperoxy epidioxides, and 55% from dihydroperoxides of methyl linolenate decomposed at 40 C. GC profiles obtained with oxidative dimers of methyl linolenate were complex and included about 20 components. Major volatile components are compared in Table 1.

Dimers from autoxidized methyl linolenate and linolenate hydroperoxides showed similar volatile decomposition product profiles with more 2,4-heptadienal and 2,4,7-decatrienal from the autoxidized linolenate dimer. Methyl 9-oxononanoate, the major decomposition product, was 63–72% from hydroperoxide dimers and 47%

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Abbreviations: GC, gas chromatograph; GC-MS, gas chromatography-mass spectrometry.

TABLE 1

Capillary Gas Chromatographic Analysis of Products from Thermal Decomposition of Monomers and Dimers from Autoxidized Methyl Linolenate and Its Oxidation Products (Relative Percentage)

	Autox Ln[a]	Hydroperoxides		OOH Epidioxides[b]		Dihydroperoxides	
Major volatiles	Dimers	Monomers	Dimers	Monomers	Dimers	Monomers	Dimers
Propanal	13.5	8.3	11.9	19.7	16.2	9.3	10.7
1-Penten-3-ol	0.0	0.6	0.6	0.5	0.0	0.0	0.0
2-Pentenal	1.7	3.2	0.7	0.7	0.0	0.0	1.3
2/3-Hexenal	1.8	2.7	1.6	0.2	0.4	0.8	1.2
2,4-Heptadienal[c]	8.0	17.3	0.7	11.0	2.2	4.1	2.2
Me heptanoate	0.7	0.4	0.7	0.0	0.0	0.4	0.0
Me octanoate	9.6	11.6	5.5	1.2	2.6	8.5	7.2
Et furanone	1.3	0.0	2.0	0.0	0.0	2.9	1.5
Epoxy heptenal	1.0	0.9	2.1	0.6	0.3	0.9	0.6
2,4,7-Decatrienal[c]	4.1	29.1	0.6	1.9	0.0	0.0	0.0
Me 8-oxo-octanoate	1.3	1.7	3.2	1.0	2.6	2.0	2.5
Me 9-oxo-nonanoate	46.9	18.3	63.3	57.9	68.2	61.8	61.4
Me Furan octanoate[c]	3.6	0.8	3.4	1.5	5.8	5.2	3.9
Me 10-oxo-8-decenoate	2.2	1.8	1.3	0.6	0.6	1.3	3.1
Me 11-oxo-9-undecenoate	4.3	3.1	2.4	3.4	1.0	2.8	4.4

[a]Autox Ln, autoxidized methyl linolenate (peroxide value 1003).

[b]OOH Epidioxides, hydroperoxy epidioxides.

[c]Separated *cis,trans* and *trans,trans* isomers are combined.

from dimers of autoxidized methyl linolenate. 2-Pentenal, 2,4-heptadienal and 2,4,7-decatrienal were found in much higher amounts from monomeric hydroperoxides than from the corresponding dimers obtained at 40 C. Methyl 9-oxononanoate, on the other hand, was found in much higher levels from dimers than the corresponding monomers.

When the volatiles from monomeric hydroperoxy epidioxides were compared with the volatiles from the corresponding dimers, significant differences were found in major components. More 2,4-heptadienal was found in the monomers than the dimers. The monomers produced 2% 2,4,7-decatrienal, but the dimers did not produce this aldehyde. More methyl 9-oxononanoate was formed from the dimers than the monomers. The major volatiles from linolenate dihydroperoxides, including propanal, methyl octanoate and 9-oxononanoate, were similar in the monomers and dimers. As with the monohydroperoxides, more 2,4-heptadienal was formed from the dihydroperoxide monomers than the corresponding dimers (Table 1). Minor volatile thermal decomposition products included 1-penten-3-ol from linolenate monohydroperoxide dimers; 2/3-hexenal, epoxy heptenal, methyl 8-oxo-octanoate, furan octanoate, 10-oxo-8-decenoate and 11-oxo-9-undecenoate from the other oxidative dimers (Table 1).

All volatiles listed in Table 1, except ethyl furanone, previously were found among thermal decomposition products from monohydroperoxides (15), hydroperoxy epidioxides and dihydroperoxides of methyl linoleate and linolenate (18,19). Ethyl furanone is a unique volatile compound detected among the decomposition products of autoxidized methyl linolenate, and the oxidative dimers of linolenate mono- and dihydroperoxides. This compound, referred to in the literature as the lactone of 4-hydroxy-2-hexenoic acid, previously was identified in thermally oxidized trilinolein (20,21), and had the same mass spectral peaks as those reported by Tressl et al. (22). In another study, monomeric 9,16-dihydroperoxide of methyl linolenate was decomposed at 160 C and only methyl octanoate and methyl 9-oxononanoate were observed by GC with a packed Carbowax column (23).

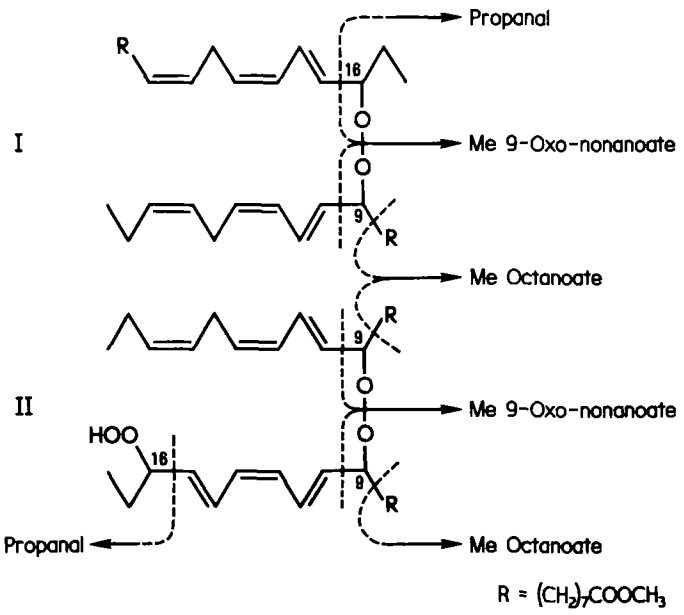
DISCUSSION

In this study, we found significant differences in volatile products generated from monomers and dimers derived from the decomposition of autoxidized methyl linolenate, its monohydroperoxides and other secondary oxidation products. The origin of volatile cleavage products observed in this work can be explained by considering mechanisms for the thermal decomposition of methyl linolenate hydroperoxides and secondary oxidation products (16,18,19) in terms of the dimeric structures established for the oxidation products of methyl linolenate (13).

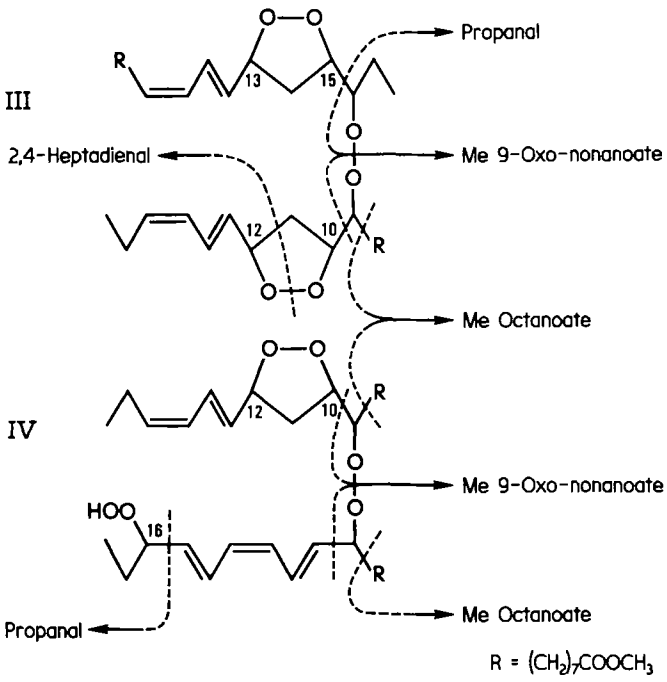
The principal cleavage mechanisms recognized for methyl linolenate hydroperoxides involve homolytic scission on each side of the hydroperoxide group (16,24,25). With the 9-hydroperoxide isomer (ranging from 30–35%), cleavage on each side of the 9-OOH group produces methyl 9-oxononanoate and decatrienal plus methyl octanoate as main decomposition products. With the 12-hydroperoxide isomer (ranging from 10–13%), cleavage on one side of the hydroperoxide forms 2,4-heptadienal. With the 13-hydroperoxide isomer (ranging from 11–13%), the same cleavage produces 3-hexenal, which readily isomerizes into a mixture of 2- and 3-isomers. With the 16-hydroperoxide isomer (ranging from 41–52%), this cleavage forms propanal as an important volatile product (16). The hydroperoxy epidioxides of methyl linolenate cleave like the monohydroperoxides and form similar products, which include methyl 9-oxononanoate, methyl octanoate and 2,4-heptadienal from the 9-hydroperoxy

epidioxide, and propanal from the 16-hydroperoxy epidioxide (18). In addition to these volatile products, 2,3-pentanedione, which is reported to have a strong butter-like smell, also has been observed among the decomposition products of the 16-hydroperoxy epidioxide of methyl linolenate (23). From our studies of volatile decomposition products, we concluded that the cyclic peroxides and other secondary products of methyl linolenate are important precursors of volatile compounds that can contribute to the flavor and oxidative deterioration of edible fats (18).

The predominant dimeric structures previously identified from methyl linolenate autoxidized at 40 C possessed structural features of the monomeric components, including monohydroperoxide, hydroperoxy epidioxide and dihydroperoxide (13). This study showed that the dimers were mixtures involving monohydroperoxides, dihydroperoxides and hydroperoxy epidioxides condensed mainly through peroxide (88%) and through ether and/or carbon linkages (12%). The major volatile decomposition products expected from different peroxide-linked dimer structures (I-IV) are summarized in Schemes 1 and 2. Cleavage between the peroxide link and the olefinic side of the 9- and 16-hydroperoxide groups would produce methyl 9-oxononanoate from dimers I and II (Scheme 1). This type of cleavage apparently is most favored because methyl 9-oxononanoate is the most important thermal volatile decomposition product observed in this study. Cleavage on the opposite side of the peroxide links accounts for the formation of methyl octanoate on one side and propanal on the other side of the first monomer unit of dimer structure I. Dimer structure II would undergo cleavage on the right to produce methyl 9-oxonoanoate and methyl octanoate, and cleavage on the left to produce propanal (Scheme 1). With both structures I and II, cleavage does not appear to take place to form 2,4,7-decatrienal from the 9-hydroperoxide isomer as it does



SCHEME 1



SCHEME 2

in the monohydroperoxide (16). Alternatively, this polyunsaturated aldehyde may be polymerized under our conditions of decomposition.

Scheme 2 shows cleavages expected from the two dimers III and IV containing hydroperoxy epidioxides, and dihydroperoxide components, cross-linked through either 9- or 16-hydroperoxide groups. Methyl 9-oxononanoate is formed by cleavage between peroxide linkages and propanal by cleavage on the 16-peroxide linkage in dimer III and the 16-hydroperoxide group in dimer IV. Methyl octanoate would be formed by cleavage of the alkyl substituents on the opposite side of the peroxide linkage on carbon-9 of these dimer structures. Cleavage across the epidioxide rings of the cyclic peroxide components in dimers III and IV would account for the formation of 2,4-heptadienal. The relatively small amounts of this compound observed (Table 1) suggest, however, that either this cleavage is minor or that this polyunsaturated aldehyde is decomposed under our conditions. 2,4-Heptadienal was also a minor product of the dimers of hydroperoxy epidioxides and dihydroperoxides. The origin of minor volatile decomposition products may be explained by the same mechanism discussed in the literature for autoxidized methyl linolenate and the monohydroperoxides and corresponding secondary oxidation products (16,24-26). Ethyl furanone may be derived from dimers containing hydroperoxide or peroxide linkage on carbon 13, by hydrogen abstraction followed by reaction with oxygen and cyclization.

This work showed that oxidative dimers of methyl linolenate and its oxidation products form volatile decomposition products related to the monomeric hydroperoxide precursors. Therefore, we believe that dimers like the monomer precursors may be important sources of volatile compounds contributing to flavor and oxidative deterioration of fats.

REFERENCES

1. Johnson, O.C., Chang, S.S., and Kummerow, F.A. (1953) *J. Am. Oil Chem. Soc. 30,* 317-332.
2. Chang, S.S., and Kummerow, F.A. (1953) *J. Am. Oil Chem. Soc. 30,* 403-407.
3. Witting, L.A., Chang, S.S., and Kummerow, F.A. (1957) *J. Am. Oil Chem. Soc. 34,* 470-473.
4. Frankel, E.N., Evans, C.D., and Cowan, J.C. (1960) *J. Am. Oil Chem. Soc. 37,* 418-424.
5. Artman, N.R. (1969) *Adv. Lipid Chem. 7,* 245-330.
6. Perkins, E.G. (1976) *Rev. Franc. Corps Gras 23,* 257-262.
7. Schieberle, P., Tsoukalas, B., and Grosch, W. (1979) *Z. Lebensm Unters. Forsch. 168,* 448-456.
8. Miyashita, K., Fujimoto, K., and Kaneda, K. (1982) *Agric. Biol. Chem. 46,* 751-755.
9. Miyashita, K., Fujimoto, K., and Kaneda, K. (1982) *Agric. Biol. Chem. 46,* 2293-2297.
10. Miyashita, K., Fujimoto, K., and Kaneda, K. (1982) *Agric. Biol. Chem. 48,* 2511-2515.
11. Miyashita, K., Hara, N., Fujimoto, K., and Kaneda, K. (1985) *Lipids 20,* 578-587.
12. Miyashita, K., Hara, N., Fujimoto, K., and Kaneda, K. (1985) *Agric. Biol. Chem. 49,* 2633-2640.
13. Neff, W.E., Frankel, E.N., and Fujimoto, K. (1988) *J. Am. Oil Chem. Soc. 65,* 616-623.
14. Frankel, E.N., Neff, W.E., Selke, E., and Brooks, D.D. (1987) *Lipids 22,* 322-327.
15. Neff, W.E., Frankel, E.N., and Weisleder, D. (1981) *Lipids 16,* 439-448.
16. Frankel, E.N., Neff, W.E., and Selke, E. (1981) *Lipids 16,* 279-285.
17. Selke, E., Rohwedder, W.K., and Dutton, H.J. (1977) *J. Am. Oil Chem. Soc. 54,* 62-67.
18. Frankel, E.N., Neff, W.E., and Selke, E. (1983) *Lipids 18,* 353-357.
19. Frankel, E.N., Neff, W.E., and Selke, E. (1984) *Lipids 19,* 790-800.
20. Chang, S.S., Peterson, R.J., and Ho, C-T. (1978) *J. Am. Oil Chem. Soc. 55,* 718-727.
21. Thompson, J.A., May, W.A., Paulose, M.M., Peterson, R.J., and Chang, S.S. (1978) *J. Am. Oil Chem. Soc. 55,* 897-901.
22. Tressl, R., Bahri, D., Holzer, M., and Kossa, T. (1977) *J. Agric. Food Chem. 25,* 459-463.
23. Peers, K.E., Coxon, D.T., and Chan, W.-S. (1984) *Lipids 19,* 307-313.
24. Frankel, E.N. (1980) *Prog. Lipid. Res. 19,* 1-22.
25. Frankel, E.N. (1983) *Prog. Lipid. Res. 22,* 1-33.

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Fluorescent Substances Derived from the Reaction of 13-Monohydroperoxylinoleic Acid and Methylamine

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Reaction of 13-monohydroperoxylinoleic acid and methylamine in the presence of ferric ions produced at least four fluorescent substances. They were separated by high performance liquid chromatography with a reverse phase column, and major fluorescent substances 1 and 3 were isolated. Fluorescent substances 1 and 3 showed the same fluorescence spectra with excitation maxima at 360 nm and emission maxima at 430 nm, and the same ultraviolet spectra with absorption maxima at 275 and 360 nm. Fluorescence of 1 and 3 was lost in alkaline media owing to the instability of the fluorophores. The fluorophores of 1 and 3 were destroyed on treatment with borohydride. The fluorescence characteristics of 1 and 3 were similar to those of the age-related fluorescent substances produced in tissues. They were different from those of the fluorescent substances derived from malonaldehyde and resembled those of the fluorescent substances derived from monofunctional aldehydes.
Lipids 23, 299–303 (1988).

Fluorescent lipofuscin granules develop in aging cells or tissues (1,2). Several researchers have isolated and analyzed fluorescent lipofuscin pigments in various tissues (3–7). The isolated lipofuscin pigments showed fluorescence with excitation maxima at 345–395 nm and emission maxima at 420–490 nm (3–7), and the fluorescence was quenched in alkaline media (5,7). Accumulation of the fluorescent lipofuscin pigments has been assumed to be due to lipid oxidation in cells or subcellular organisms (8,9). Although it has been shown that oxidized lipids or fatty acids give rise to fluorescence by interaction with primary amines or proteins (10–20), the structures and the properties of the fluorophores produced in these reactions are not known. Only a few preliminary studies showing the formation of multiple fluorescent substances in the reaction of mixed fatty acid hydroperoxides have appeared (14,17,18).

In this study, we investigated the formation of fluorescent substances in the reaction of 13-monohydroperoxylinoleic acid (13-LOOH) and methylamine as a model for fluorescent lipofuscin pigment formation in tissues. The fluorescent products were purified, and their fluorescence characteristics were elucidated. The fluorescence characteristics of the products were compared with those of the fluorescent lipofuscin pigments and the fluorescent substances derived from malonaldehyde or monofunctional aldehydes.

MATERIALS AND METHODS

Materials. 13-Monohydroperoxylinoleic acid (13-LOOH) was prepared according to the method of Gardner (21) by use of linoleic acid (grade III, Sigma Chemical Co., St. Louis, MO) and lipoxydase (type 1: 130,000 units/mg, Sigma Chemical Co.). Purity of the hydroperoxide estimated on the basis of ultraviolet absorption coefficient at 234 nm: 24,500 (22) was 95.1%. High performance liquid chromatography (HPLC) with a straight phase column of the sample after methylation with diazomethane revealed that it was consisted of *trans,trans* and *cis,trans* isomers (23).

Analysis. Ultraviolet absorption spectra were measured with a Hitachi 323 recording spectrophotometer. Fluorescence spectra were measured with a Hitachi 204 fluorescence spectrophotometer. Relative fluorescence intensity was expressed as the percentages of the intensity of 0.1 μM quinine sulfate in 0.1 N sulfuric acid (excitation at 353 nm and emission at 445 nm). Silicic acid (Mallinkcrodt, St. Louis, MO) column chromatography was performed with the solvent system chloroform/methanol (3:1). A Sephadex LH-20 column was used for the removal of salts with 0.017% acetic acid/methanol/acetonitrile (3:1:1) as solvent.

HPLC was performed on a Shimadzu liquid chromatograph fitted with a stainless steel column packed with Unisil QC-18 5 μm (16.7 × 300 mm) or LiChrosorb RP-18 5 μm (4.6 × 25 mm). The columns were eluted with a mobile phase composed of 8.3 mM $(NH_4)_2HPO_4$ (pH 3.5)/methanol/acetonitrile (3:1:1, v/v/v) at a flow rate of 3.5 or 0.8 ml/min, respectively. Fluorescent peaks were detected by use of a Hitachi fluorescence monitor with excitation at 360 nm and emission at 440 nm. Ultraviolet absorbing peaks were detected by use of a Hitachi wavelength turnable effluent monitor.

Mass spectra were taken with a JEOL JMS DX300 instrument equipped with a fast atom bombardment (FAB) ion source and a DA5000 data system. The sample was dissolved in methanol and placed on a stainless steel probe target with glycerol matrix. FAB/MS was operated with xenon gas at an emission current of 20 mA and an accelerating voltage of 6 KV in the positive mode.

RESULTS

A solution containing 2 mM 13-LOOH and 200 mM methylamine in methanol/water was incubated at 37 C in the absence and presence of ferric ions. Time course studies of the fluorescence intensities of the reaction mixtures (Fig. 1) showed that the formation of fluorescence was gradual in the absence of ferric ions, and was accelerated in the presence of ferric ions. Degradation of 13-LOOH by ferric ions promoted fluorescence formation. The reaction mixtures exhibited fluorescence with an excitation maximum at 363 nm and an emission maximum at 428 nm in methanol. The reaction mixtures of 13-LOOH alone did not produce any significant fluorescence irregardless of whether ferric ions were present.

Isolation of the fluorescent substances was attempted. A mixture containing 20 mM 13-LOOH, 2 M methylamine and 0.1 mM ferric ions was incubated at 37 C for

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Abbreviations: FAB, fast atom bombardment; HPLC, high performance liquid chromatography.

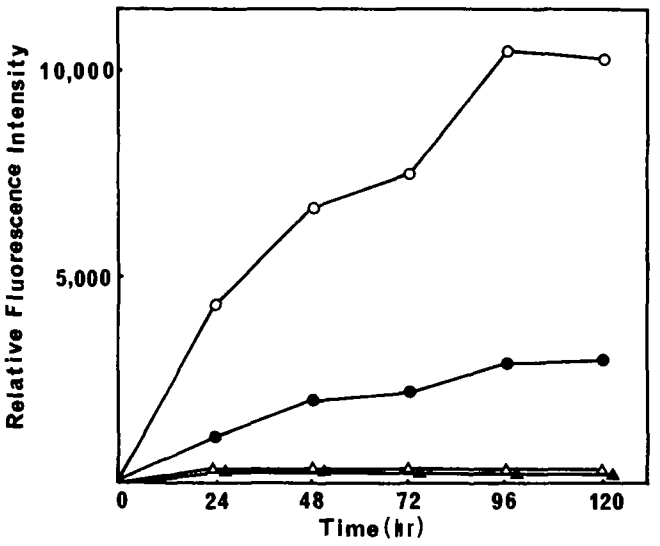

FIG. 1. Time course of the formation of fluorescence in the reaction of 13-LOOH and methylamine. A mixture of 2 mM 13-LOOH, 200 mM methylamine hydrochloride and 0.1 mM ferric chloride (○), 2 mM 13-LOOH and 200 mM methylamine hydrochloride (●), 2 mM 13-LOOH and 0.1 mM ferric chloride (△) or 2 mM 13-LOOH alone (▲) in methanol/water (1:1) adjusted at pH 5.5 by addition of 1 N NaOH was incubated at 37 C. Fluorescence spectra of the mixtures were taken after 100-fold dilution in methanol. Relative fluorescence intensities measured at the excitation (363 nm) and emission (428 nm) maxima were expressed as the percentages of the intensity of 0.1 μM quinine sulfate in 0.1 N sulfuric acid.

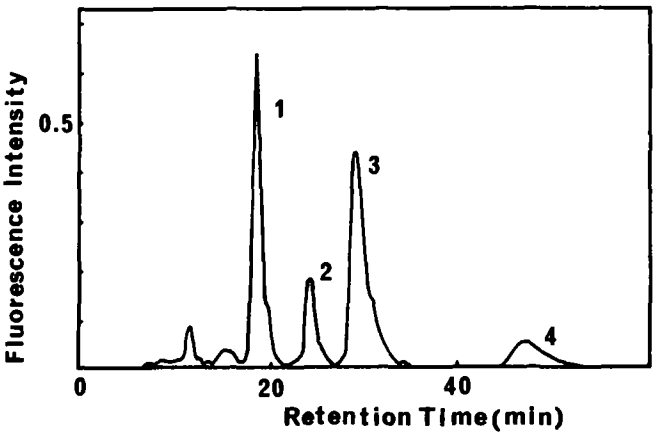

FIG. 2. HPLC of the fluorescent substances derived from the reaction of 13-LOOH and methylamine. A 10 ml mixture of 20 mM 13-LOOH, 2 M methylamine hydrochloride and 0.1 mM ferric chloride in methanol/water (1:1) adjusted at pH 5.5 was shaken at 37 C for 120 hr. The mixture was evaporated to dryness, and the residue was applied to a silicic acid (25 g) column. The fluorescent eluate was collected and evaporated to dryness. The residue was subjected to HPLC using a Unisil QC-18 5 μm column at a flow rate of 3.5 ml/min. The peaks were detected with excitation at 360 nm and emission at 440 nm.

120 hr. The reaction mixture was passed through a column of silicic acid to remove most of the salts. The fluorescence spectrum of the eluate was unchanged during column chromatography, and 80% of the fluorescence was recovered (Table 1). The fluorescent substances were then separated by HPLC using a preparative reverse phase column (Fig. 2). Four fluorescent peaks were detected, and each fraction was collected. The fluorescence spectra of the fractions were the same as the spectrum of the reaction mixture, indicating that the fluorescent substances were unchanged by HPLC (Table 1). Forty-three percent of fluorescence of the reaction mixture was recovered in the four peaks.

The fluorescent eluate from the silicic acid column was dissolved in methanol, ethanol, 2-propanol or chloroform and kept at room temperature for up to 20 hr. HPLC of the solutions revealed that none of the fluorescent substances was degraded in the solvents. The fluorescent eluate was dissolved in the buffered solutions of various pH values and kept for up to 20 hr. HPLC of the solutions revealed that the fluorescent substances 1, 2 and 3 were unchanged in the solutions at pH 4.9 and 7.0, but they were degraded in the solutions at pH 10.0 and 11.5 (Fig. 3). The intensities of the peaks diminished to less than 20% after 20 hr at pH 10.0 and after five hr at pH

TABLE 1

Fluorescence Spectra and Recovery of the Fluorescent Substances Derived from the Reaction of 13-LOOH and Methylamine

Samples	Fluorescence[a]			
	Excitation maximum (nm)	Emission maximum (nm)	Relative fluorescence intensity[b]	Recovery (%)
Reaction mixture[c]	363	428	198,000	100
Silicic acid column chromatography[c]	360	425	158,000	80
HPLC[c]				
1	363	425	27,000	14
2	364	425	12,000	6
3	363	425	31,000	16
4	370	423	13,000	7

[a]Spectra were measured in methanol.

[b]Relative fluorescence intensity was based on 10 ml of the solution and expressed as percentages of the intensity of 0.1 μM quinine sulfate.

[c]The same as described in the legend to Figure 2.

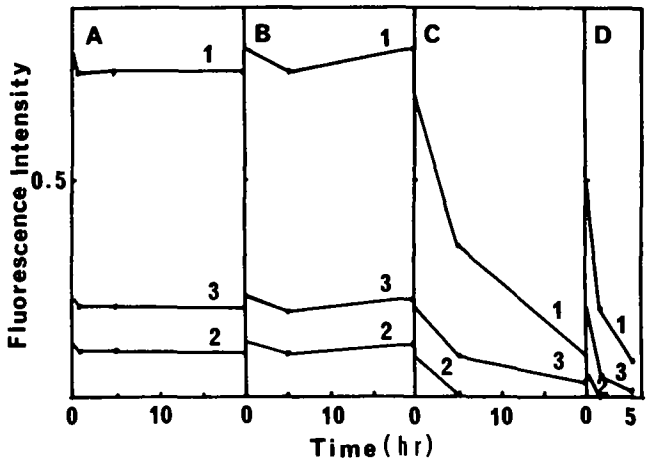

FIG. 3. Stability of fluorescent substances 1, 2, and 3 at various pH values. The fluorescent eluate of the silicic acid column was evaporated to dryness and dissolved in the buffer. The solutions were kept at room temperature for up to 20 hr and analyzed by HPLC with a LiChrosorb RP-18 5 μm column at a flow rate of 0.8 ml/min. 0.05 M citrate buffer, pH 4.9 (A); 0.07 M phosphate buffer, pH 7.0 (B); 0.05 M borate buffer, pH 10.0 (C); and 0.025 M borate buffer, pH 11.5 (D).

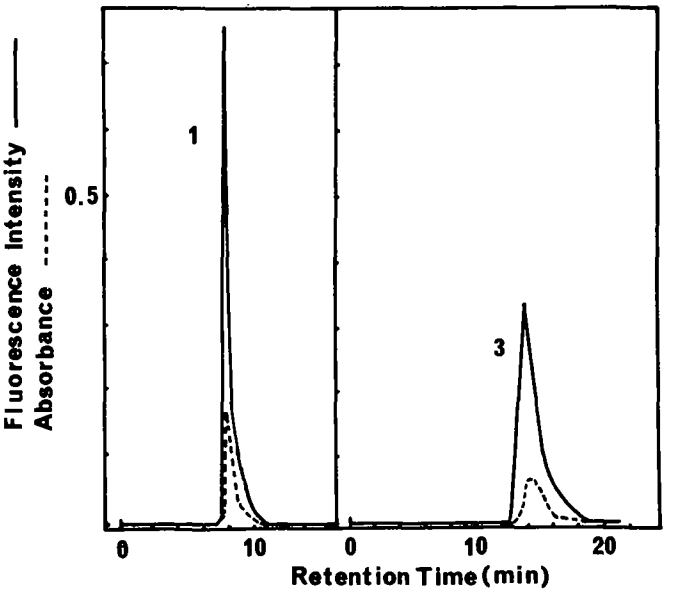

FIG. 4. HPLC of the purified samples of 1 and 3. Fluorescent substances 1 and 3 obtained in Figure 2 were rechromatographed, and the fluorescent fractions were desalted through a Sephadex LH-20 column. The fractions of 1 and 3 were analyzed with a LiChrosorb RP-18 5 μm column at a flow rate of 0.8 ml/min. The peaks were detected by fluorescence with excitation at 360 nm and emission at 440 nm, and by ultraviolet absorption at 360 nm.

11.5. The initial fluorescence intensities of the eluate could not be recovered when the eluate was kept at pH 11.5 for five hr and subsequently adjusted to pH 4.9.

Two major fluorescent substances, 1 and 3, were rechromatographed by HPLC with the same preparative column, and the salts in the eluates were removed by passing through a Sephadex LH-20 column. Fluorescent substances 1 and 3 thus purified showed single fluorescent and ultraviolet absorbing peaks at retention times of 8.0 min and 14.5 min, respectively, when analyzed by HPLC with an analytical reverse phase column (Fig. 4).

Purified samples of 1 and 3 in methanol showed similar fluorescence spectra with an excitation maximum at 360 nm and an emission maximum at 430 nm, and similar ultraviolet absorption spectra with absorption maxima at 275 and 360 nm (Fig. 5). Fluorescence intensities of the purified samples of 1 and 3 in methanol (adsorbance at 360 nm: 1.0) relative to 0.1 μM quinine sulfate were 48,000% and 47,000%, respectively. In order to elucidate the structures of the compounds, mass spectra were measured by using a fast atom bombardment ion source. However, no reproducible mass fragmentation patterns were obtained because of the presence of impurities without fluorescence or ultraviolet absorption. The structures of the compounds could not be elucidated.

Fluorescence spectra of the purified samples of 1 and 3 showed little change in methanol, ethanol, 2-propanol, chloroform and water, but the fluorescence intensities were different (Table 2). While 1 and 3 exhibited intensities in ethanol, 2-propanol and water comparable to those in methanol, much lower intensities were observed in chloroform.

Fluorescence intensities of 1 and 3 were little influence in methanol/1 N HCl, but they were greatly lowered in methanol/1 N NaOH (Table 2). This loss in fluorescence is due to the instability of 1 and 3 in alkaline media. The fluorescence intensities of 1 and 3 in methanol/10 mM borohydride (pH 9.2) also were lowered. This decrease

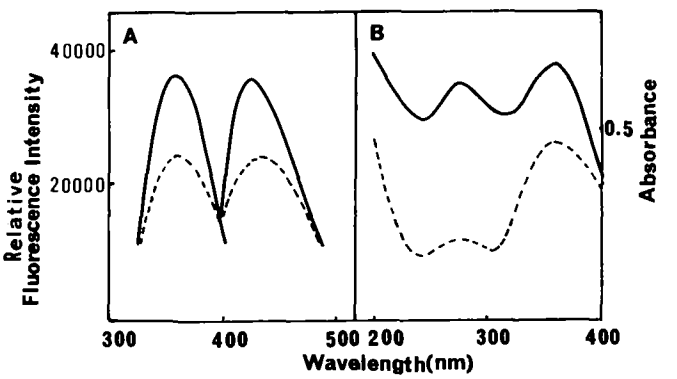

FIG. 5. Fluorescence (A) and ultraviolet absorption (B) spectra of the purified samples of 1 (——) and 3 (– – –) in methanol. Relative fluorescence intensities of the solutions assessed for ultraviolet absorption spectra were expressed as the percentages of the intensity of 0.1 μM quinine sulfate.

could not be accounted for by the high pH values because the intensities in methanol/10 mM NaOH (pH 9.4) retained about half of the intensities observed at neutral pH. The fluorophores of 1 and 3 were destroyed on treatment with borohydride.

DISCUSSION

Previously, we have shown that linoleic, linolenic and arachidonic acids produced fluorescent substances with excitation maxima at 355–370 nm and emission maxima at 420–440 nm by reaction with methylamine under aerobic conditions (18). The fluorescence was influenced

TABLE 2

Fluorescence Spectra and Intensities of the Purified Samples of 1 and 3 under Various Conditions

Solvent	1			3		
	Excitation maximum (nm)	Emission maximum (nm)	Intensity[a]	Excitation maximum (nm)	Emission maximum (nm)	Intensity[a]
Methanol	360	430	100	357	430	100
Ethanol	358	430	139	358	430	108
2-Propanol	362	434	162	357	428	101
Chloroform	350	430	15	358	422	44
Water	357	430	117	356	428	99
Methanol/1 N HCl (9:1)	360	430	93	357	430	94
Methanol/1 N NaOH (9:1)	380	420	12	380	420	9
Methanol/10 mM NaOH (9:1)[b]	360	420	42	370	420	57
Methanol/10 mM $NaBH_4$ (9:1)[c]	380	405	7	385	435	8

[a]Relative intensity was expressed at the excitation and emission maxima.
[b]pH 9.4.
[c]pH 9.2.

little in acidic media but was affected differently in alkaline media with the fatty acids. The characteristics of the fluorescent substances indicate that they differ in structure depending on fatty acids from which they are derived.

In this investigation, the fluorescent substances in the reaction of 13-LOOH with methylamine were isolated, and their fluorescence characteristics were clarified. The fluorescent substances were produced during degradation of the hydroperoxide, and at least four components were produced. Fluorescence spectra of the products were similar and had excitation maxima at 360 nm and emission maxima at 430 nm. The products were degraded in alkaline solutions, and thus the fluorescence intensities were diminished greatly. This is in good agreement with the observations that the fluorescence of the isolated lipfuscin of human and rat testes was quenched in alkaline media (5,7). The fluorophores in these products were destroyed upon treatment with borohydride.

Malonaldehyde, one of the degradation products of the hydroperoxide, is a possible candidate for the production of the fluorophores. It reacts with methylamine, primary amines or proteins to produce fluorescence due to 1,4-dihydropyridine-3,5-dicarbaldehyde moieties under close to physiological conditions (24-28). These fluorophores revealed the longer wavelengths in fluorescence excitation (385-405 nm) and emission (440-470 nm) and the higher fluorescence intensities. Fluorescence intensities of these compounds in methanol (absorbance 1.0) relative to 0.1 μM quinine sulfate was 50,000-150,000% (27,28). The fluorescence of the compounds was quenched in acidic media but not in alkaline media, and the fluorophores were destroyed upon treatment with borohydride (27,28). The fluorescence characteristics of 1 and 3 were different from those of the fluorophores derived from malonaldehyde.

Monofunctional aliphatic aldehydes that also are produced during the degradation of the hydroperoxide can form fluorescent substances by reaction with primary amines (29,30) or proteins (13,19,20,31), which exhibited excitation maxima at 340-370 nm and emission maxima at 400-440 nm. Fluorescence spectra of 1 and 3 are similar to those of these fluorescent substances. Fluorescence intensities of 1 and 3 are close to those of these compounds: 300-30,000% (30). However, these compounds resisted borohydride reduction, and the fluorescence intensities were quenched in the aqueous solutions through a wide pH range (30). While the fluorescence spectra of 1 and 3 were similar to those of the fluorophores produced from the monofunctional aliphatic aldehydes, their fluorescence characteristics were not identical in every respect.

13-LOOH produced several fluorescent substances by reaction with methylamine. The fluorescence characteristics of the substances were similar to those of the fluorescent lipofuscin pigments with respect to maximum wavelengths and loss of fluorescence in alkaline media. These substances were distinguishable from the fluorescent products derived from malonaldehyde and resembled those derived from monofunctional aldehydes.

REFERENCES

1. Strehler, B.L. (1964) in *Advances in Gerontology Research* (Strehler, B.L., ed.) Vol. 1, pp. 343-384, Academic Press, New York, NY.
2. Elleder, M. (1981) in *Age Pigments* (Sohal, R.S., ed.) pp. 203-241, Elsevier/North-Holland Biomedical Press, Amsterdam.
3. Siakotos, A.N., and Koppang, N. (1973) *Mech. Age Develop. 2,* 177-200.
4. Taubold, R.D. (1975) *Lipids 1,* 383-390.
5. Trombly, R., Tappel, A.L., Coniglio, J.G., Grogan, W.M. Jr., and Rhamy, R.K. (1975) *Lipids 10,* 591-596.
6. Csallany, A.S., and Ayaz, K.L. (1976) *Lipids 11,* 412-417.
7. Shimasaki, H., Ueta, N., and Privett, O.S. (1980) *Lipids 15,* 236-241.
8. Chio, K.S., Reiss, R., Fletcher, B., and Tappel, A.L. (1969) *Science 166,* 1535-1536.
9. Dillard, C.J., and Tappel, A.L. (1971) *Lipids 6,* 715-721.
10. Dillard, C.J., and Tappel, A.L. (1973) *Lipids 8,* 183-189.
11. Bidlack, W.R., and Tappel, A.L. (1973) *Lipids 8,* 203-207.
12. Braddock, R.J., and Dugan, L.R. Jr. (1973) *J. Am. Oil Chem. Soc. 50,* 343-347.
13. Fletcher, B.L., and Tappel, A.L. (1971) *Lipids 6,* 172-175.
14. Shimasaki, H., Privett, O.S., and Hara, I. (1977) *J. Am. Oil Chem. Soc. 54,* 119-123.

15. Shimasaki, H., Ueta, N., and Privett, O.S. (1982) *Lipids 17,* 878-883.
16. Tabata, T., Yoden, K., Takei, H., and Iio, T. (1979) *Yakugaku Zasshi 99,* 711-716.
17. Yoden, K., Iio, T., and Tabata, T. (1981) *Yakugaku Zasshi 101,* 437-442.
18. Kikugawa, K., Watanabe, S., and Kurechi, T. (1984) *Chem. Pharm. Bull. 32,* 638-645.
19. Kikugawa, K., Takayanagi, K., and Watanabe, S. (1985) *Chem. Pharm. Bull. 33,* 5437-5444.
20. Beppu, M., Murakami, K., and Kikugawa, K. (1986) *Chem. Pharm. Bull. 34,* 781-788.
21. Gardner, H.W. (1975) *Lipids 10,* 248-252.
22. Gardner, H.W. (1970) *J. Lipid Res. 11,* 311-321.
23. Chan, H.W.S., and Prescott, F.A.A. (1975) *Biochim. Biophys. Acta 380,* 141-144.
24. Kikugawa, K. (1986) *Adv. Free Rad. Biol. Med. 2,* 389-416.
25. Kikugawa, K., Maruyama, T., Machida, Y., and Kurechi, T. (1981) *Chem. Pharm. Bull. 29,* 1423-1432.
26. Kikugawa, K., Machida, Y., Kida, M., and Kurechi, T. (1981) *Chem. Pharm. Bull. 29,* 3003-3011.
27. Kikugawa, K., and Ido, Y. (1984) *Lipids 19,* 600-608.
28. Kikugawa, K., Ido, Y., and Mikami, A. (1984) *J. Am. Oil Chem. Soc. 61,* 1574-1581.
29. Yoden, K., Matsuzaki, R., Iio, T., and Tabata, T. (1982) *Yakugaku Zasshi 102,* 768-773.
30. Kikugawa, K., and Sawamura, A. (1987) *J. Am. Oil Chem. Soc. 64,* 1156-1162.
31. Tashiro, Y., Okitani, A., Utsunomiya, N., Kaneko, S., and Kato, H. (1985) *Agric. Biol. Chem. 49,* 1739-1747.

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Adenylate Cyclase and β-Receptors in Salivary Glands of Rats Fed Diets Containing *Trans* Fatty Acids

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The effects of *trans* fatty acids on adenylate cyclase were determined in the submandibular salivary glands (SMSG) of rats fed diets containing 20% corn oil, 20% partially hydrogenated soybean oil (PHSBO) or 18% PHSBO + 2% corn oil. The fluoride- and isoproterenol-stimulated adenylate cyclase activities were higher in the SMSG from rats fed 20% PHSBO than in the control group fed 20% corn oil. The feeding of 2% corn oil with the diet containing 18% PHSBO resulted in a complete restoration of isoproterenol-stimulated and a partial restoration of fluoride-stimulated adenylate cyclase activity. There was no significant difference in the concentration of the β-adrenergic receptor or in the receptor-binding affinity constants among the three dietary groups as measured by [^{3}H]dihydroalprenolol (DHA)-binding. Higher fluorescence polarization of diphenyl hexatriene (DPH) was observed in SMSG membranes of rats fed diet containing 20% PHSBO than in the other two oils, suggesting that membrane fluidity may play a role in adenylate cyclase activity.

Lipids 23, 304-308 (1988).

Partially hydrogenated vegetable oils contain a number of positional and geometric isomers of unsaturated fatty acids. Like the naturally occurring *cis*-isomers, the *trans* fatty acids also can be incorporated into various tissues of animals (1) and man (2). Studies from our laboratory have shown that dietary *trans* fatty acids can be incorporated into various lipids of the submandibular salivary glands (SMSG) (3) and in the lacrimal glands (4). The fluorescence polarization of diphenylhexatriene (DPH) was higher in plasma membranes of the exocrine glands of rats fed diets containing partially hydrogenated soybean oil (PHSBO) than corn oil, suggesting a lower membrane fluidity in the former group. Since membrane fluidity is considered to be important in controlling a number of physiological phenomena, including the activities of some membrane-associated enzymes (5,6), we have been investigating the effects of feeding diets containing *trans* fatty acids on the activity of adenylate cyclase (ATP pyrophosphate lyase [cyclizing], EC 4.6.1.1) in different tissues. An increase in fluoride-stimulated adenylate cyclase activity was observed in membranes of the lacrimal glands from rats fed 20% PHSBO (4). Because of the physiological importance of adenylate cyclase in the secretory mechanisms of the salivary glands, we have studied the effects of feeding diets containing *trans* fatty acids on adenylate cyclase in the SMSG. By using [^{3}H]dihydroalprenolol-binding analysis, the characteristics of β-adrenergic receptors as affected by feeding diets containing *trans* fatty acids also were studied.

MATERIALS AND METHODS

All the biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclic [^{3}H]AMP (41 Ci/mmol), α-[^{32}P]ATP (410 Ci/mmol) and [^{3}H]dihydroalprenolol (55 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). All the dietary ingredients were purchased from TEKLAD (Madison, WI). PHSBO was supplied by Kraft Inc. (Glenview, IL).

Feeding study. Three groups of male weanling Sprague-Dawley rats (Holtzman Co., Madison, WI), 16-20/group, were fed ad libitum semipurified diets containing 20% corn oil (CO) (group 1), 20% PHSBO (group 2) or 18% PHSBO + 2% CO (group 3). The composition of the basal diet was similar to that of AIN-76A (7,8). It contained (in %) casein (vitamin-free), 20.0; DL-methionine, 0.3; sucrose, 50.0; oil (corn oil, PHSBO or 18% PHSBO + 2% CO), 20.0; cellulose, 5.0; mineral mixture (AIN-76A), 3.5; vitamin mixture (AIN-76A), 1.0; choline bitartrate, 0.2 and butylated hydroxytoluene, 0.002. The fatty acid composition of the oils was determined by gas chromatography and is shown in Table 1. PHSBO contained about 48% of the total fatty acids as *trans* octadecenoic acid (t-18:1). Rats were housed individually in suspended stainless steel cages in a temperature- and light-controlled room (22-23 C, 12-hr light-dark cycle). Rats had free access to water and feed and were weighed at least once a week. Diets were prepared fresh every two to three weeks and were stored at 4 C.

Membrane preparation. Rats were killed by decapitation after 16 or 18 wk of feeding the diets. The SMSG

TABLE 1

Fatty Acid Composition of the Oils

Fatty acid	Corn oil	PHSBO	18% PHSBO + 2% corn oil
14:0	—	0.2	0.2
16:0	11.0 ± 0.2^{a,b}	10.5 ± 0.1^{a}	11.2 ± 0.1^{b}
16:1	0.3	0.2	0.2
18:0	2.1 ± 0.1^{a}	13.1 ± 1.5^{b}	14.6 ± 0.1^{b}
t-18:1	—^{a}	47.8 ± 1.8^{b}	38.5 ± 0.1^{c}
c-18:1	26.9 ± 0.5^{a}	23.9 ± 0.6^{b}	26.9 ± 0.1^{a}
t,c-18:2*	—^{a}	1.9 ± 0.1^{b}	0.9 ± 0.4^{a}
c,c-18:2	59.1 ± 0.7^{a}	2.0 ± 0.1^{b}	7.9 ± 0.1^{c}
18:3	0.7 ± 0.1	—	—

Values are area percentage, average of triplicate analyses (mean ± SEM). Values with different superscript letters (a, b and c) in the same row are significantly different ($P < 0.05$) using analysis of variance, Newman-Keul's test.

*Includes some c,t and t,t; PHSBO, partially hydrogenated soybean oil; —, not detectable.

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Abbreviations: DHA, dihydroalprenolol; DPH, diphenyl hexatriene; EFA, essential fatty acids; PHSBO, partially hydrogenated soybean oil; SMSG, submandibular salivary glands; TLC, thin layer chromatography.

were dissected out, separated from the sublingual glands, rinsed with physiological saline and weighed. To obtain sufficient samples for analyses, at each time point (16 and 18 wk) the SMSG were pooled from four to five rats within each dietary group for the preparation of plasma membranes for adenylate cyclase activity determinations and for lipid analyses. A similar number of rats was used for the preparation of plasma membranes for studies on the binding of [^{3}H]dihydroalprenolol and for fluorescence polarization studies. Plasma membranes were prepared by the differential centrifugation method of Durham et al. (9) as described (10). Membranes were stored under liquid nitrogen until assayed.

Plasma membranes and homogenates of SMSG were assayed for 5′-nucleotidase (11) and Mg^{2+}-ATPase (12), marker enzymes for plasma membranes. Mitochondrial and microsomal contamination of the membranes was assessed by measuring succinic dehydrogenase (13) and glucose-6-phosphatase (14) activities, respectively. Protein content was measured by the method of Lowry et al. (15) using bovine serum albumin as standard.

[^{3}H]Dihydroalprenolol (DHA) binding. [^{3}H]DHA-binding was measured by incubating 0–29 nM [^{3}H]DHA with 250 μg of the membrane protein for 15 min at 37 C in the presence of 50 mM Tris buffer, pH 7.4, and 10 mM $MgCl_2$ in a final volume of 0.5 ml. The reaction was terminated by immersing in ice the tubes containing the reaction mixture. This was followed by rapid filtration on GF/A glass fiber filters (Whatman Lab Sales, Inc., Hillsboro, OR). The residue was washed with 3 × 4 ml of the ice-cold incubation buffer. The filters were transferred to scintillation vials, dried at room temperature, 10 ml of liquid scintillation fluid (Ready-Solv, EP, Beckman) was added, and the radioactivity was counted using a Liquid Scintillation Spectrometer (Beckman LS 6800, Fullerton, CA).

[^{3}H]DHA-binding to membranes from each dietary group was measured in duplicate in three independent experiments per group. For each concentration of labeled DHA, a companion "nonspecific" incubation in the presence of 1 mM unlabeled DHA was conducted. All binding data then were analyzed using the LIGAND-PC programs (1986, version 2.3.10) (16) on a Zenith 171 personal computer. Data files for each experiment were prepared using SCAHOT, and the three data files per group were analyzed simultaneously using SCAFIT. SCAFIT uses weighted nonlinear least-squares curve-fitting to estimate binding parameters (affinity constant, dissociation constant, receptor capacity and nonspecific binding estimate). Each group of data was subjected to both one-site and two-site model analysis, using the automatic initial parameter estimates provided by SCAFIT.

Adenylate cyclase activity. Adenylate cyclase activity was determined in triplicate samples of the membranes (50 μg protein) by monitoring the conversion of α-[^{32}P]ATP to cyclic [^{32}P]AMP using the procedure of Sinensky et al. (17). The product, cyclic [^{32}P]AMP, was separated from the unreacted substrate by sequential column chromatography using Dowex and alumina columns according to the method of Salomon et al. (18). To monitor the recovery of cyclic [^{32}P]AMP, cyclic [^{3}H]AMP was added to the samples after incubation and before column chromatography. The details of the procedure have been described (10). Stimulated enzyme activity was measured in the presence of 15 mM sodium fluoride or 20 μM isoproterenol. The basal activity was measured in the absence of fluoride or isoproterenol.

Fluorescence Polarization Studies. Membranes were labeled by incubating them at 37 C for one hr with a solution of DPH in phosphate-buffered saline as described (3), and fluorescence polarization was measured in a SLM fluorescence polarization spectrophotometer (SLM 4800) according to the procedure of Shinitzky and Inbar (19) and Shinitzky and Barenholz (20).

Fatty acid composition. Total lipids were extracted from aliquots of the SMSG membranes using Bligh and Dyer's method (21). Phospholipids were separated by column chromatography (22). Methyl esters were prepared from the dietary lipids and the SMSG membrane phospholipids using borontrifluoride-methanol (23). Fatty acid methyl esters were analyzed by argentation thin layer chromatography (TLC) and gas chromatography as described (3). The fatty acid composition of the dietary oils also was determined by using a similar method as described (3).

Statistical analyses. There was no significant difference in adenylate cyclase activity or [^{3}H]DHA-binding in the membranes prepared after 16 or 18 wk of feeding. Therefore, the data from 16 and 18 wk were combined and are presented as mean ± SE. The differences in means among the three dietary groups were analyzed using analysis of variance, Newman-Keul's test (24).

RESULTS

Rats fed 20% PHSBO had a lower growth rate than that of the control group fed 20% CO (Table 2). The incorporation of 2% CO in the PHSBO diet resulted in a partial restoration of the rate of growth. The weights of the SMSG followed a pattern similar to that of the body weights among the three dietary groups. When expressed on the basis of 100 g of body weights, however, the gland weights essentially were the same in the three groups. These values were (mg SMSG/100 g body wt, mean ± SE): 64.5 ± 2.2 in 20% CO group, 65.2 ± 0.6 in 20% PHSBO group and 60.8 ± 2.5 in 18% PHSBO + 2% CO group.

TABLE 2

Body Weight Gains of Rats Fed Diets Containing PHSBO

	Weeks on diets			
Diet	4	8	12	16
20% CO	202.2^a ±3.4	380.4^a ±9.2	473.3^a ±13.6	539.3^a ±26.6
20% PHSBO	176.5^b ±3.1	302.6^b ±5.8	366.9^b ±6.6	398.3^b ±9.6
18% PHSBO + 2% CO	188.2^b ±4.5	343.6^c ±9.5	437.8^a ±7.6	492.9^a ±13.7

Values are in g, mean ± SEM of 16–20 rats in each group. Values with different superscripts in the same column are significantly different from each other ($P < 0.01$) using analysis of variance, Newman-Keul's test. The initial body weight was 77.7 ± 1.6 g.

CO, corn oil; PHSBO, partially hydrogenated soybean oil.

There was no significant difference in the purity of the plasma membranes among the three dietary groups as evaluated by marker enzymes (5'-nucleotidase and Mg^{2+}-ATPase for plasma membranes, succinic dehydrogenase for mitochondria and glucose-6-phosphatase for microsomes). The specific activity of 5'-nucleotidase was the same in the three dietary groups (20% corn oil, group 1, 20.4 ± 2.4; 20% PHSBO, group 2, 19.6 ± 2.9; 18% PHSBO + 2% corn oil, group 3, 20.5 ± 1.9). Similarly, Mg^{2+}-ATPase activity was also the same (94.9 ± 2.3 in group 1, 98.6 ± 5.9 in group 2 and 98.2 ± 5.4 in group 3). The values are mean ± SEM, μmol Pi per mg protein/hr. Mitochondrial contamination was about 3% as measured by succinic dehyrogenase activity. There was some glucose-6-phosphatase activity in the membranes, indicating a cosedimentation of microsomes with the plasma membranes fraction. However, there was no difference in glucose-6-phosphatase activity among the three dietary groups.

Adenylate cyclase activity in plasma membranes of rats fed the various diets is shown in Table 3. There was no difference in the basal activity. However, fluoride-, and isoproterenol-stimulated adenylate cyclase activity and fold stimulation was the highest in membranes of the SMSG of rats fed 20% PHSBO. When 2% PHSBO was replaced by an equivalent amount of CO, the adenylate cyclase activity in the SMSG membranes was similar to that of the control group fed 20% CO. However, the fold-stimulation of adenylate cyclase by fluoride only partially was restored to that of the control levels.

The mean specific binding of [^{3}H]DHA to the SMSG plasma membranes of rats from the three dietary groups is shown in Figure 1. Specific binding for each determination was calculated (total − nonspecific = specific) using the nonspecific binding value estimated in LIGAND, which ranged from 13.8 to 17.8% of total bound counts and averaged 15.9% ± 0.42 (SEM). There was no apparent difference in the saturation curves for the three groups. The binding parameters obtained from LIGAND analysis of the data are shown in Table 4. No significant difference in either adrenergic receptor capacity or binding affinity was caused by the dietary treatments.

Trans 18:1, the main component constituting about 48% of the total fatty acids in PHSBO, was incorporated into the membrane phospholipids (Table 5). The levels of c,c-18:2 and 20:4 were lower in the SMSG membrane phospholipids of rats fed 20% PHSBO than in those fed 20% corn oil. Also, 20:3 ω-9 constituted about 4–5% of the total fatty acids in the phospholipids of membranes of rats fed 20% PHSBO. Only trace amounts of this fatty acid were present in the other two groups. The ratio of 20:3 ω-9/20:4, an established index of essential fatty acids (EFA) deficiency (25), was >0.4 in the SMSG membrane phospholipids of rats fed 20% PHSBO. This ratio and the

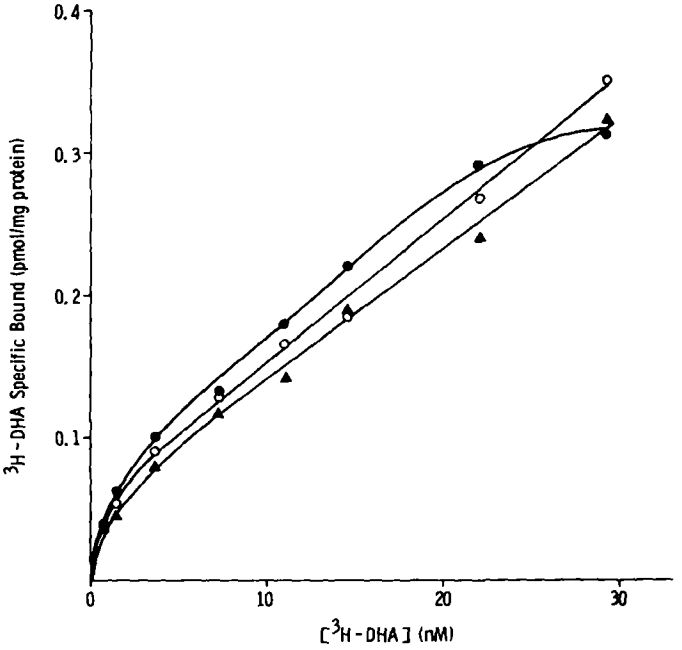

FIG. 1. Specific [^{3}H]DHA-binding to the SMSG membranes of rats fed different diets: 20% CO ●—●, 20% PHSBO ○—○, 18% PHSBO + 2% CO ▲—▲. Nonspecific binding, as estimated by LIGAND (16) analysis, was subtracted from total binding data. Each data point is the average of three assays, each done in duplicate, and the abscissa values are the total [^{3}H]DHA concentrations. Nonspecific binding was 15.9% ± 0.42 of total bound (mean ± SEM).

TABLE 3

Basal, Fluoride- and Isoproterenol-activated Adenylate Cyclase Activity in SMSG Plasma Membranes of Rats Fed PHSBO

Diet	Basal	+Fluoride	+Isoproterenol
20% CO	6.7 ± 0.8^a (1.00)	23.0 ± 1.8^a (3.43)	13.3 ± 1.2^a (1.99)
20% PHSBO	6.5 ± 1.0^a (1.00)	33.4 ± 2.6^b (5.14)	19.1 ± 1.9^b (2.94)
18% PHSBO + 2% CO	6.0 ± 0.7^a (1.00)	26.9 ± 2.5^{a,b} (4.48)	12.7 ± 1.4^a (2.12)

Enzyme activities are expressed as pmol cAMP formed/mg protein/min. Values are mean ± SEM of eight to nine assays per group; each assay was done in triplicate.

Values with different superscripts in the same column are significantly different from each other ($P < 0.05$) using analysis of variance, Newman-Keul's test. Fold-stimulation is shown in parentheses.

Fluoride, 15 mM; isoproterenol, 20 μM; CO, corn oil; PHSBO, partially hydrogenated soybean oil.

TABLE 4

Binding Parameters for [^{3}H]DHA-binding to SMSG Membranes From Rats Fed PHSBO

Diet	K_A ($M^{-1} \times 10^{-8}$)	[Ro] (pmol/mg protein)	K_D (nM)
20% Corn oil	2.01 ± 0.27	0.282 ± 0.023	4.97 ± 0.66
20% PHSBO	2.02 ± 0.25	0.263 ± 0.020	4.95 ± 0.60
18% PHSBO + 2% CO	1.97 ± 0.26	0.232 ± 0.020	5.07 ± 0.67

Values (mean ± SEM) were determined using LIGAND, simultaneously analyzing the data from three separate experiments for each individual group. The one receptor-site model thereby was determined the most appropriate for the data. None of the differences among groups is significant by either one-way analysis of variance or Student-Newman-Keul's test.

fatty acid patterns were restored to the control values (20% corn oil group) when 2% corn oil was fed with 18% PHSBO.

The fluorescence polarization of DPH is shown in Figure 2. Higher P-values were observed in membranes of rats fed 20% PHSBO compared with the other two groups. Fluorescence polarization was higher, especially at temperature above 20 C, in membranes of rats fed 18% PHSBO + 2% corn oil than in membranes of rats fed 20% corn oil.

DISCUSSION

The lipid dependence of adenylate cyclase has been reported in a number of studies (26-28). There are very few studies, however, on the effects of *trans* fatty acids upon the activities of adenylate cyclase. In one published report, an increase in adenylate cyclase activity (basal, fluoride-, and PGE_1-stimulated) was reported in membranes from mouse LM cells grown in a medium supplemented with *trans* octadecenoate (29). This observation is consistent with our present findings of an increase in fluoride-, and isoproterenol-stimulated adenylate cyclase activity in the SMSG and similar findings in the lacrimal glands (4) of rats fed 20% PHSBO.

The adenylate cyclase system consists of three essential components: membrane receptor, guanine-binding regulatory proteins (G_s and G_i) and the catalytic unit of adenylate cyclase. Theoretically, any of these components may be altered by nutritional modifications. Because neither the concentration of β-adrenergic receptor nor its [^{3}H]DHA-binding were significantly affected by feeding 20% PHSBO, it appears that the observed increase in isoproterenol-stimulated adenylate cyclase activity in plasma membranes of SMSG of rats fed 20% PHSBO was not due to any modifications in β-adrenergic receptor. An increase in fluoride- and isoproterenol-stimulated activity, therefore, may be due to the possible changes in the levels of guanine nucleotide binding regulatory proteins, an increase in the stimulatory proteins (G_s) or a decrease in the inhibitory proteins (G_i). Fluoride is known to activate adenylate cyclase by direct interaction with the G proteins (30-32). It also is possible, however, that the characteristics (K_m or V_{max}) of the catalytic subunit of adenylate cyclase may be altered as a result of feeding diets containing 20% PHSBO.

Our results on [^{3}H]DHA-binding to the rat SMSG membranes showed a single binding site, an observation that is consistent with our previous findings (33) and with those of Bylund et al. (34) and Cutler et al. (35). Also, the affinity of the binding sites and their density was similar to that previously reported by us (33) and by these investigators (34,35). However, there was no significant effect of *trans* fatty acids on the characteristics of radioligand binding. We have found that the feeding of diets supplemented with 1-2% cholesterol resulted in an increase in the affinity of the [^{3}H]DHA-binding sites and a decrease in β-adrenergic receptor content in the rat SMSG (33).

TABLE 5

Fatty Acid Composition of Total Phospholipids of SMSG Membranes of Rats Fed Diets Containing PHSBO

Fatty acid	Diet fed		
	20% CO	20% PHSBO	18% PHSBO + 2% CO
14:0	2.0 ± 0.1[a]	3.6 ± 0.3[b]	2.3 ± 0.3[a]
16:0	21.3 ± 0.7[a]	18.0 ± 0.7[b]	18.2 ± 0.6[b]
16:1	—	5.4	4.5
18:0	13.6 ± 0.3[a]	10.9 ± 0.4[b]	13.3 ± 0.6[a]
t-18:1	—[a]	9.2 ± 0.3[b]	8.9 ± 0.5[b]
c-18:1	11.8 ± 1.0[a]	26.0 ± 2.2[b]	15.2 ± 0.9[a]
t,c-18:2*	—	1.6	0.6
c,c-18:2	12.2 ± 0[a]	4.1 ± 0.9[b]	8.6 ± 0.8[c]
18:3	1.0 ± 0.1[a]	1.3 ± 0.2[a]	0.8 ± 0.2[a]
20:2	0.9 ± 0.1[a]	2.0 ± 0.3[b]	0.7 ± 0.1[a]
20:3 ω-9	—[a]	4.4 ± 0.3[b]	—[a]
20:3 ω-6	3.2 ± 0[a]	0.2 ± 0.2[b]	2.2 ± 0.1[c]
20:4	20.7 ± 0.7[a]	9.3 ± 0.3[b]	18.5 ± 0.1[c]
20:5	3.0 ± 0.4[a]	0.8 ± 0.2[b]	1.0 ± 0.1[b]
22:4	3.0 ± 0.6[a]	0.6 ± 0.3[b]	1.4 ± 0.2[b]
22:5	2.1 ± 0.3[a]	0.9 ± 0.1[b]	1.2 ± 0[b]
22:6	1.9 ± 0.1[a]	3.2 ± 0.3[b]	2.1 ± 0.3[a]

Values are area percentage, average of triplicate analyses within each group (mean ± SEM).

Values with different superscript letters (a, b and c) in the same row are significantly different ($P < 0.05$) using analysis of variance, Newman-Keul's test.

c, fatty acids containing *cis* double bonds; t, fatty acids containing a *trans* double bond; —, not detectable.

*c,t or t,t.

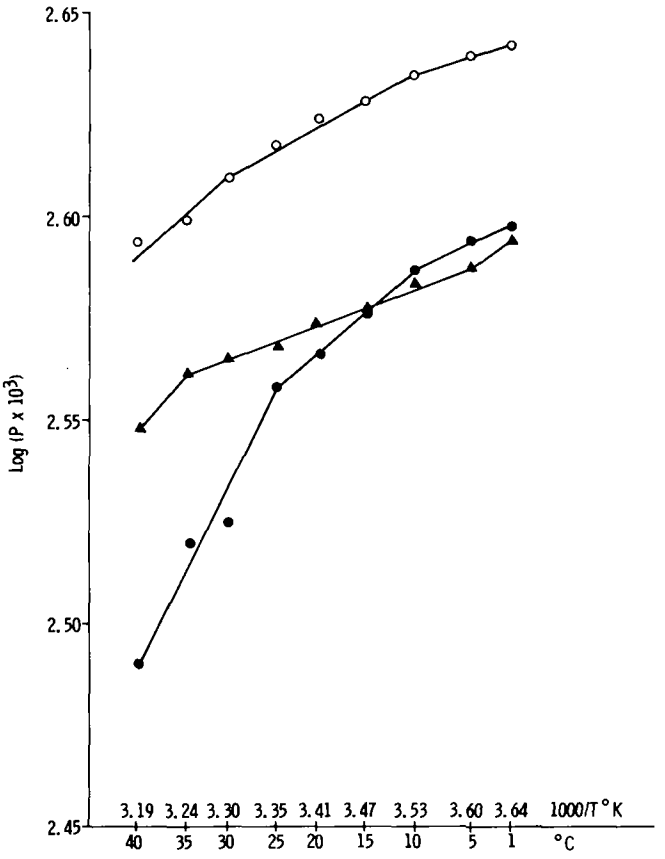

FIG. 2. Fluorescence polarization of DPH in plasma membranes from SMSG of rats fed different diets: 20% CO ●—●, 20% PHSBO O—O, 18% PHSBO + 2% CO ▲—▲.

The rats fed 20% PHSBO were deficient in EFA as indicated by reduced body weight gains, an increase in the levels of 20:3 ω-9 fatty acid in plasma and SMSG and a ratio of 20:3 ω-9/20:4, which was >0.4 in the SMSG membrane phospholipids and in plasma. A 20:3 ω-9 to 20:4 ratio greater than 0.4 in tissues and plasma is considered an established index of EFA deficiency (25). With the replacement of 2% of the PHSBO with corn oil, there was no biochemical evidence of EFA deficiency as evaluated by the above criteria. In a previous study (10), we also have observed an increase in fluoride-stimulated adenylate cyclase activity in SMSG membranes of rats fed an EFA-deficient or a marginally EFA-deficient diet. An increase in adenylate cyclase activity in our previous study (10) was associated with a decrease in membrane fluidity as shown by higher fluorescence polarization of DPH. Since our present results and previous findings (3) show that the feeding of *trans* fatty acid-containing diet such as 20% PHSBO to rats resulted in their incorporation into the SMSG lipids and in a decrease of membrane fluidity, it appears reasonable to conclude that the observed increase in adenylate cyclase activity may be associated with an EFA deficiency induced by feeding a diet containing high levels of *trans* fatty acids and deficient in EFA. Only 2.0% of the total fatty acids were contributed by linoleic acid (18:2) in the 20% PHSBO diet. On caloric basis, 0.8% of the total calories were derived from 18:2, which makes it somewhat deficient in EFA because the minimum requirements of EFA for growing rats are 1-2% of the total calories (25). Dietary *trans* fatty acids have been shown to intensify EFA deficiency (36-38). In the 18% PHSBO + 2% CO diet, 3% of the total calories were derived from 18:2, thus making it adequate with respect to the EFA requirements. The 20% corn oil diet contained about 23% of the total calories as 18:2.

In another recent study (33), we have observed that adenylate cyclase activity, the concentration of β-adrenergic receptor and membrane fluidity as measured by the fluorescence polarization of DPH were decreased in membranes prepared from rats fed cholesterol-supplemented diets. Since the membrane fluidity was affected in the same direction, i.e. decreased in the two nutritional models, whereas adenylate cyclase activity was altered in an opposite direction (increased by feeding *trans* fatty acids and decreased by feeding cholesterol), it appears that in addition to the membrane fluidity, other more specific factors also may be important in regulating the enzyme activity. For adenylate cyclase, this may include changes in the receptor content or its affinity, a modification of the levels of the guanine nucleotide-binding regulatory proteins (G_s and G_i) and in the characteristics of the catalytic unit of adenylate cyclase. Studies along these lines are underway in our laboratory to get a better understanding of the mechanism(s) of the diet-induced changes in adenylate cyclase activity.

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REFERENCES

1. Wood, R. (1979) in *Geometric and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.) pp. 213-281, American Oil Chemists' Society, Champaign, IL.
2. Ohlrogge, J.B. (1983) in *Dietary Fats and Health* (Perkins, E.G., and Visek, W.J., eds.) pp. 359-374, American Oil Chemists' Society, Champaign, IL.
3. Alam, S.Q., Alam, B.S., and Banerji, A. (1985) *Lipids 20,* 16-23.
4. Alam, S.Q., Banerji, A., and Alam, B.S. (1985) *Current Eye Res. 4,* 1253-1262.
5. Bloj, B., Morero, R.D., Farias, R.N., and Trucco, R.E. (1973) *Biochim. Biophys. Acta 311,* 67-79.
6. Kimelberg, H.K. (1977) *Cell Surf. Rev. 3,* 205-293.
7. American Institute of Nutrition (1977) *J. Nutr. 107,* 1340-1348.
8. American Institute of Nutrition (1980) *J. Nutr. 110,* 1726.
9. Durham, J.P., Galanti, N., and Revis, N.W. (1975) *Biochim. Biophys. Acta 394,* 388-405.
10. Alam, S.Q., and Alam, B.S. (1986) *J. Nutr. 116,* 1620-1630.
11. Aronson, N.N., and Touster, O. (1974) in *Methods Enzymol. 31,* pt. A, 90-102.
12. Jorgensen, P.L. (1974) in *Methods Enzymol. 32,* pt. B (Fleischer, S. and Packer, L., eds.) pp. 277, Academic Press, New York, NY.
13. Pennington, R.J. (1961) *Biochem. J. 80,* 649-654.
14. DeDuve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Applemans, F. (1955) *Biochem. J. 60,* 604-617.
15. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
16. Munson, P.J., and Rodbard, D. (1980) *Anal. Biochem. 107,* 220-239.
17. Sinensky, M., Minneman, K., and Molinoff, P.B. (1979) *J. Biol. Chem. 254,* 9135-9141.
18. Salomon, Y., Londos, C., and Rodbell, M. (1974) *Anal. Biochem. 58,* 541-548.
19. Shinitzky, M., and Inbar, M. (1974) *J. Mol. Biol. 85,* 603-615.
20. Shinitzky, M., and Barenholtz, Y. (1974) *J. Biol. Chem. 249,* 2652-2657.
21. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
22. Rouser, G., Kritchevsky, G., and Yamamoto, A. (1967) in *Lipid Chromatographic Analysis* (Marinetti, G.V., ed.), Vol. 1, pp. 118-120, New York, NY.
23. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600-608.
24. Snedecor, T.W., and Cochran, W.J. (1967) in *Statistical Methods,* 6th edn., pp. 273-275, Iowa State University Press, Ames, IA.
25. Holman, R.T. (1960) *J. Nutr. 70,* 405-410.
26. Lefkowitz, R.J. (1975) *J. Mol. Cell. Cardiol. 7,* 27-37.
27. Levey, G.S. (1971) *J. Biol. Chem. 246,* 7406-7410.
28. Limbird, L.E., and Lefkowitz, R.J. (1976) *Mol. Pharmacol. 12,* 559-567.
29. Englehard, V.H., Glaser, M., and Storm, D.R. (1978) *Biochemistry 17,* 3191-3200.
30. Howlett, A.C., and Gilman, A.G. (1980) *J. Biol. Chem. 255,* 2861-2866.
31. Downs, R.W. Jr., Spiegel, A.M., Singer, M., Reen, S., and Aurbach, G.D. (1980) *J. Biol. Chem. 255,* 949-954.
32. Kaslow, H.R., Johnson, G.L., Brothers, V.M., and Bourne, H.R. (1980) *J. Biol. Chem. 255,* 3736-3741.
33. Alam, S.Q., Ren, Y-F., and Alam, B.S. (1987) *J. Dent. Res. 66,* 605-607.
34. Bylund, D.B., Forte, L.R., Morgan, D.W., and Martinez, J.R. (1981) *J. Pharmacol. Exp. Ther. 218,* 134-141.
35. Cutler, L.S., Boccuzzi, J., Yaeger, L., Bottaro, B., Christian, C.P., and Martinzez, J.R. (1981) *Virchows Archiv [Pathol Anat] 392,* 185-198.
36. Hill, E.G., Johnson, S.B., and Holman, R.T. (1979) *J. Nutr. 109,* 1759-1766.
37. Hwang, D.H., and Kinsella, J.E. (1979) *Prostaglandins 17,* 543-559.
38. Kinsella, J.E., Bruckner, G., Mai, J., and Shimp, J. (1981) *Am. J. Clin. Nutr. 34,* 2307-2318.

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The Influence of n-6 and n-3 Fatty Acids on Kidney Phospholipid Composition and on Eicosanoid Production in Aging Rats

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Changes in eicosanoid production may contribute to some of the complications of the aging process such as atherosclerosis and glomerular sclerosis. Polyunsaturated fatty acids of the n-6 and n-3 series are precursors of eicosanoids. We fed diets containing safflower oil as a source of n-6 fatty acids, fish oil as a source of n-3 fatty acids or beef tallow as a source of saturated fats to three groups of normal rats from 2–18 months of age. We demonstrated incorporation of the n-3 fatty acids, 20:5n-3 and 22:6n-3 into kidney phospholipids. Feeding of the diet containing n-3 fatty acids was associated with a markedly decreased glomerular production of PGE, 6-keto-$PGF_{1\alpha}$ and TXB_2. It also decreased the aortic production of 6-keto-$PGF_{1\alpha}$ and platelet production of TXB_2. No significant effect of n-6 fatty acids on dienoic eicosanoid production was observed. There were no adverse effects on kidney function as measured by urinary protein excretion and serum creatinine levels or on renal morphology by any diet. A diet enriched in n-3 fatty acids for 18 months remains effective in decreasing dienoic eicosanoids in the aging rat.
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The spontaneous occurrence of glomerular sclerosis in aging rats (1) and humans (2,3) is well documented. Senescent glomerular sclerosis correlates with extra and intrarenal vascular changes (4–6) or may result from the same pathogenetic factors that determine the development of atherosclerosis such as changes in plasma lipids (7), platelet function (8) or hemodynamic factors. Altered eicosanoid production may have a pathogenetic role in both atherosclerosis and glomerular sclerosis.

Dietary polyunsaturated fatty acids (PUFA) are incorporated into tissue phospholipids and are precursors of eicosanoids. This experiment was conducted to study the influence of substituting n-6 or n-3 PUFA for saturated fats in the diet of normal rats for a prolonged period. We determined the fatty acid composition of kidney phospholipids and dienoic eicosanoid production by kidney glomeruli, platelets and aorta.

METHODS

Animals and diets. Two-month-old female Wistar rats (Harlan Labs, Indianapolis, IN) were divided into three groups and fed three different diets from 2–18 months of age. These diets (TEKLAD, Madison, WI) differed only in their fat composition; they contained 200 g/kg of protein as vitamin-free casein and were in compliance with current nutritional recommendations (9). The saturated fatty acid (SFA) diet provided 190 g/kg of beef tallow and 10 g/kg of soybean oil. The n-6 PUFA diet contained 200 g/kg safflower oil (TEKLAD), and the n-3 PUFA diet contained 200 g/kg of MaxEPA (registered trademark of Seven Seas Health Care, Ltd., Hull, England); MaxEPA is a concentrated blend of fish oils from which vitamin A and D have been removed and to which vitamin E (0.5 mg/g) has been added. An antioxidant (BHA 26 mg/kg) was added to all diets. The vitamin mix used was Teklad #40060, providing 0.24 g/kg diet of dry vitamin E acetate. The n-3 PUFA diet therefore contained 0.1 g/kg more vitamin E than the other two diets. The mineral mix used was AIN-76, the composition of which has been reported (9). The major fatty acids of the dietary fats analyzed by gas chromatography are shown in Table 1. Rats were fed a measured amount of the SFA diet (approximately 20 g) every day. Rats in the other two groups received the same volume of food, but due to the higher weight per volume of the n-6 and n-3 PUFA compared to the SFA diets, rats fed these diets had approximately 12% more food available. At 16 months of age, blood pressure was measured by the tail cuff method in unanesthetized animals in restraining cages; urine was collected by placing rats in metabolic cages for 16 hr without food but with water freely available. At 18 months of age, rats were killed under ketamine-xylazine anesthesia. Blood was withdrawn by cardiac puncture. Kidneys were perfused in vivo with Hank's balanced salt solution (HBSS, Gibco, Grand Island, NY), removed and decorticated. Kidney samples were stored at −20 C for phospholipid fatty acid determinations. Other tissue samples were fixed in Mossman's formalin fixative for light microscopy. Renal glomeruli were isolated, and a segment of the thoracic aorta was excised to measure prostaglandin production.

Kidney phospholipid fatty acid composition. For this analysis, samples of tissue were selected randomly from each dietary group. Kidney tissue was homogenized in

TABLE 1

Fatty Acids in Dietary Fats

Fatty acid	Saturated	n-6 PUFA	n-3 PUFA
12:0	2.1	2.0	—
14:0	3.4	—	—
16:0	24.9	6.6	16.9
16:1	2.6	—	10.5
18:0	18.7	2.4	3.6
18:1	40.0	11.0	18.1
18:2	4.5	76.7	3.6
18:3n-6	—	—	2.8
18:3n-3	—	—	2.2
20:4n-6	—	—	2.5
20:5n-3	—	—	18.8
22:5n-3	—	—	2.8
22:6n-3	—	—	12.2

Values are percentage of total peak area.

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Abbreviations: ANOVA, analysis of variance; GC, gas chromatograph; HBSS, Hank's balanced salt solution; PAS, periodic acid Schiff; PG, prostaglandin; PUFA, polyunsaturated fatty acid; RIA, radioimmunoassay; SFA, saturated fatty acid.

saline and extracted with chloroform/methanol (10). Total lipids were separated using a silica SEP PAK cartridge (Waters, Milford, MA) with the phospholipid fraction eluting with methanol (11). Phospholipids were methylated in boron trichloride in methanol at 90 C for one hr under nitrogen. Methyl esters of fatty acids were separated on a Supelcowax 10 wide-bore capillary column, using polyethylene glycol as a stationary phase (Supelco, Bellefonte, PA). Prepurified helium, at 12 ml/min was used as the carrier gas. A 5790A Hewlett Packard gas chromatograph (GC) was used with an injector temperature of 210 C. The GC was interfaced with a 3390A Hewlett Packard integrator. Fatty acids were identified with a combination of PUFA 1 and PUFA 2 standards (Supelco, Bellefonte, PA) and 18:3, 24:0 and 24:1 (NuChek Prep., Elysian, MN). All reagents were HPLC-grade (Fisher, Cincinnati, OH).

Glomerular eicosanoids. Kidneys were perfused in vivo with HBSS and heparin (2 U/ml). Glomeruli were isolated and pooled from kidneys of two rats using a previously reported sieving technique (12) and were determined microscopically to be about 80% pure and about 95% decapsulated. Glomeruli were incubated in a total volume of 0.5 ml in HBSS, containing 0.1% BSA (Fraction V, Sigma Chemical Co., St. Louis, MO) pH 7.4, at 37 C for one hr. Samples then were centrifuged immediately and supernatants saved for prostaglandin (PG) radioimmunoassay (RIA). Glomerular pellets were saved for protein determination by the Lowry method. For RIA, tritiated prostaglandins and scintillation cocktail were purchased from New England Nuclear (Boston, MA), and standards and antibodies were obtained from Seragen, Inc. (Boston, MA). The smallest detectable doses of PG standard on the linear portion of the standard curves were 4.7 pg for PGE, 12.0 pg for 6-keto-$PGF_{1\alpha}$ and 5.2 pg for TXB_2. Nonspecific binding was always less than 3.0%. Intra- and interassay coefficients of variation were 2.7% and 5.5%, respectively, for all assays.

Aortic 6-Keto-$PGF_{1\alpha}$ production. This was measured in the incubation media by RIA after segments of aorta were incubated in HBSS and 0.1% BSA (pH 7.4) at 37 C for one hr in a total volume of 1.0 ml. Tissue was air-dried and weighed and the results expressed as ng/mg tissue.

Platelet TXA_2 production. This was measured after clotting whole blood at 37 C for 30 min with subsequent centrifugation. TXB_2, the stable metabolite of TXA_2 was measured in serum by RIA.

Urinary protein. This was measured using the Bradford reagent (Bio Rad, Rockville Ct., NY), and plasma creatinine was determined colorimetrically using the Jaffe reaction. For light microscopy, kidney tissue was stained with periodic acid Schiff (PAS) and examined blind-coded for the presence of glomerular sclerosis or hypercellularity and tubular changes.

Statistics. Comparisons between groups were tested by one-way analysis of variance (ANOVA). Differences between individual groups were established by the Duncan's test.

RESULTS

The fatty acid composition of the kidney phospholipids for each dietary group is shown in Table 2. Rats fed n-6 PUFA had a significantly higher proportion of 18:2n-6 than rats fed SFA and reduced monounsaturated fatty acids 16:1 and 18:1. Rats fed n-3 PUFA had significantly higher proportions of 20:5n-3 and 22:6n-3 and lesser amounts of 18:2n-6 and 20:4n-6 than animals fed the SFA and n-6 PUFA diets. The levels of 18:1 were lower in the n-3 PUFA group in comparison to the SFA group but higher than those in the n-6 PUFA group.

TABLE 2

Fatty Acid Composition of Kidney Phospholipids

Fatty acid	SFA (n = 6)	n-6 PUFA (n = 5)	n-3 PUFA (n = 4)
16:0	20.6 ± 1.5	21.5 ± 5.0	23.7 ± 2.7
16:1	1.3 ± 0.4	0.2 ± 0.4**	1.7 ± 0.3
18:0	20.6 ± 2.1	23.4 ± 1.2	23.9 ± 0.6
18:1	16.6 ± 2.2	8.0 ± 0.8**	12.6 ± 1.5*
18:2n-6	10.6 ± 1.3	19.5 ± 0.9**	2.9 ± 0.5*
20:4n-6	24.9 ± 1.4	22.9 ± 2.1	13.9 ± 1.2*
20:5n-3	0.8 ± 0.6	0.7 ± 0.2	13.0 ± 1.5*
22:5n-3	2.8 ± 1.1	2.5 ± 0.6	2.5 ± 2.3
22:6n-3	0.9 ± 0.6	0.9 ± 0.7	4.5 ± 2.5*
24:1	1.1 ± 0.9	0.7 ± 0.7	2.0 ± 2.3

*$p < 0.01$ vs SFA and n-6 PUFA groups.
**$p < 0.01$ vs SFA and n-3 PUFA groups.
Values are percentage of total area ± SD.

The results of eicosanoid determinations are shown in Table 3. Compared with the rats fed SFA, rats fed n-3 PUFA had a markedly diminished gomerular production of all eicosanoids measured. The levels of aortic 6-keto-$PGF_{1\alpha}$ and platelet TXB_2 observed in the n-3 PUFA fed rats were 50% of those values found in the SFA-fed groups. The effects of n-6 PUFA diet on dienoic eicosanoid production were not statistically significant from the SFA-fed group.

Body weight, blood pressure, serum creatinine and urinary protein excretion are shown in Table 4. Rats fed n-6 and n-3 PUFA diets gained more weight than those fed the SFA diet. No significant differences between groups were observed for blood pressure, serum creatinine and urinary protein excretion. Light microscopic examination of kidney samples did not show any significant degree of glomerular sclerosis or tubular abnormalities in any of the rats.

DISCUSSION

We have demonstrated significant incorporation of n-6 and n-3 dietary PUFA into kidney phospholipids at the end of a 16-month feeding period in aging rats. These findings extend shorter-term observations by other workers (13).

We also observed a significant reduction in tissue production of dienoic eicosanoids, which are derived from linoleic acid. This inhibition was much more pronounced on the kidney glomeruli than on aorta or platelets; the suppressing effect of eicosapentaenoic acid on dienoic eicosanoids has been shown (14). Eicosapentaenoic acid competes with arachidonic acid as a substrate at the enzymatic level. Previous reports have demonstrated suppression of urinary PGE and 6-keto-$PGF_{1\alpha}$, decreased

TABLE 3

Effects of n-6 and n-3 PUFA on Eicosanoid Production by Isolated Glomeruli, Aorta and Platelets

	SFA	n-6 PUFA	n-3 PUFA	Number of observations
		Isolated glomeruli		
PGE (pg/mg prot)	1590 (770, 3280)	603 (226, 1600)	21* (11, 40)	7,7,6
6-keto-$PGF_{1\alpha}$ (pg/mg prot)	230 (91, 630)	108 (40, 287)	<20* (ND)	8,7,5
TXB_2 (pg/mg prot)	721 (323, 1580)	960 (440, 2120)	17* (10, 28)	7,7,5
		Aorta		
6-keto/$PGF_{1\alpha}$ (ng/mg tissue)	22.3 (15.4, 32.3)	18.6 (12.5, 27.5)	10.2* (6.6, 15.8)	17,11,13
		Platelets		
TXB_2 (ng/ml serum)	347 (224, 537)	339 (204, 562)	169* (132, 219)	14,12,14

*$p < 0.01$ vs SFA and n-6 PUFA groups; ND, not detectable.

Values are geometric means (−SD, +SD). Column on the right shows the number of observations for groups SFA, n-6 PUFA and n-3 PUFA, respectively.

TABLE 4

Body Weight, Blood Pressure, Serum Creatinine Levels and Urinary Protein Excretions

	SFA (n = 17)	n-6 PUFA (n = 12)	n-3 PUFA (n = 14)
Body weight[a] (g)	280 ± 26	341 ± 55*	331 ± 35*
Blood pressure[a] (mm Hg)	110.8 ± 5.9	112.9 ± 7.5	115.9 ± 10.3
S creat[b] (mg/dl)	0.44 (0.35, 0.55)	0.54 (0.32, 0.91)	0.36 (0.30, 0.44)
U_{Prot}[b] (mg/16 hr)	0.97 (0.23, 4.13)	0.34 (0.1, 3.73)	2.57 (0.92, 7.16)

*$p < 0.01$.

[a]Values are means ± SD.

[b]Values are geometric means (−SD, +SD).

6-keto-$PGF_{1\alpha}$ by kidney and decreased serum TXB_2 in rats after four wk of being fed a diet in which 40% of the daily energy intake was provided by cod-liver oil, while safflower oil had an increasing effect on serum TXB_2 only (15). MaxEPA oil at 20% energy in the diet of normal rats for six wk suppressed serum TXB_2, urinary 6-keto-$PGF_{1\alpha}$ and PGE_2, and aortic production of 6-keto-$PGF_{1\alpha}$ (16). Other reports have shown similar results feeding cod-liver oil for five wk in the dexamethasone-treated hypertensive rat (17) and the one kidney-one clip hypertensive rat (18). The present experiment has the importance of extending these observations for 16 months, demonstrating preservation of the dienoic eicosanoid suppressor effect of the fish oil-supplemented diet. We did not observe a significant effect of n-6 PUFA diet on dienoic eicosanoid production. These effects were not associated with any evidence of significant renal pathology, as evidenced by normal levels of serum creatinine and urinary protein excretion and by the absence of significant renal abnormalities by light microscopy. The levels of 18:2n-6 and 20:4n-6 were similar in tissues from the SFA and n-6 PUFA groups despite the significantly lower body weights in the SFA-fed group. These weight differences may have been due to higher food intake by rats in the n-6 and n-3 PUFA groups. It is unlikely that the weight differences had a major influence on the dienoic eicosanoid suppression found in the n-3 PUFA-fed rats.

The role of dietary factors, specifically of polyunsaturated fats in development of glomerular sclerosis and progressive renal disease, has been discussed (19,20). Several reports have shown beneficial effects of PUFA-enriched diets on the progression of immunologic (21–23) and nonimmunologic (24–28) models of renal disease in which the hallmark of progression is the development of glomerular sclerosis. It is attractive to propose that

PUFA might be a useful part of a dietary strategy to prevent glomerular sclerosis of aging. Long-chain n-3 PUFA derived from fish oil have other well-known biologic effects that may have potential benefit on development of atherosclerosis (29,30) and possibly of glomerular sclerosis.

The role of eicosanoids during the aging process is not clear. In the aorta of aging rats, investigators report decreased PGI_2 production by smooth muscle cells (31), increased PGI_2 production by aortic strips (32), decreased PGI_2 by endothelial cells in male rats, yet increased PGI_2 in smooth muscle homogenates by both male and female rats (33). In the kidney, PGI_2 formation from arachidonic acid decreases with age (34), while the ratio of urinary TXA_2 to creatinine increases with age in normal humans (35). Since PGI_2 is a vasodilator and TXA_2 a vasoconstrictor, these changes may have a pathophysiologic role in the reduction of renal function with age. We have shown that dienoic eicosanoid production can be altered by using n-3 PUFA for long periods in the diet of normal aging rats without deleterious effects on renal function or morphology.

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REFERENCES

1. Hirokawa, K. (1975) *Mech. Ageing Devel. 4,* 301-316.
2. Kaplan, C., Pasternack, B., Shah, H., and Gallo, G. (1975) *Am. J. Pathol. 80,* 227-234.
3. Kappel, B., and Olsen, S. (1980) *Virchows Arch. A. Path. Anat. Histol. 387,* 271-277.
4. Kasiske, B. (1987) *Kidney Int. 31,* 1153-1159.
5. McLachlan, M.S.F., Guthrie, J.C., Anderson, C.K., and Fulker, M.J. (1977) *J. Pathol. 121,* 65-78.
6. Hollenberg, E.N., Adams, D.F., Solomon, H.S., Rashid, A., Abrams, H.L., and Merrill, J.P. (1974) *Circ. Res. 34,* 309-316.
7. Edwards, K.D.G. (1981) *Controversies in Nephrology III,* pp. 3-19, Georgetown University/Masson Pub., New York, NY.
8. Johnson, M., Ramey, E., and Ramwell, P.W. (1975) *Nature 253,* 355-357.
9. Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies (1977) *J. Nutr. 107,* 1340-1348.
10. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
11. Juaneda, P., and Rocquelin, S. (1985) *Lipids 20,* 40-41.
12. Hassid, A., Konieczkowski, M., and Dunn, M.J. (1979) *Proc. Natl. Acad. Sci. USA 76,* 1155-1159.
13. Huang, Y.S., Nassar, B.A., and Horrobin, D.F. (1986) *Biochim. Biophys. Acta 879,* 22-27.
14. Nassar, B.A., Manku, M.S., Huang, Y.S., Jenkins, D.K., and Horrobin, D.F. (1987) *Prost. Leuk. Med. 26,* 253-263.
15. Croft, K.D., Beilin, L.J., Vandongen, R., and Mathews, E. (1984) *Biochim. Biophys. Acta 795,* 196-207.
16. Croft, K.D., Codde, J.P., Barden, A., Vandongen, R., and Beilin, L. (1985) *Biochim. Biophys. Acta 834,* 316-323.
17. Codde, J.P., and Beilin, L.J. (1985) *Clin. Sci. 69,* 691-699.
18. Codde, J.P., McGowan, H.M., Vandongen, R., and Beilin, L.J. (1985) *Hypertension 7,* 886-892.
19. Barcelli, U., and Pollak, V.E. (1985) *Nephron 41,* 209-212.
20. Klahr, S., Buerkert, J., and Purkerson, M. (1983) *Kidney Int. 24,* 579-587.
21. Prickett, J.D., Robinson, D.R., and Steinberg, A.D. (1983) *Arth. Rheum. 26,* 133-139.
22. Kelley, V.E., Ferretti, A., Izui, S., and Strom, T.B. (1985) *J. Immunol. 134,* 1914-1919.
23. Kher, V., Barcelli, U., Weiss, M., Gallon, L., Pajel, P., Laskarzewski, P., and Pollak, V.E. (1986) *Prost. Leuk. Med. 22,* 323-334.
24. Barcelli, U.O., Weiss, M., and Pollak, V.E. (1982) *J. Lab. Clin. Med. 100,* 786-797.
25. Heifets, M., Morrisey, J.J., Purkerson, M., Morrison, A.R., and Klahr, S. (1987) *Kidney Int. 32,* 335.
26. Izumi, Y., Weiner, T.W., Franco-Saenz, R., and Mulrow, P.J. (1986) *Proc. Soc. Exp. Biol. Med. 183,* 193-198.
27. Barcelli, U.O., Miyata, J., Ito, Y., Gallon, L., Laskarzewski, P., Weiss, M., Hitzemann, R., and Pollak, V.E. (1986) *Prostaglandins 32,* 211-219.
28. Ito, Y., Barcelli, U., Yamashita, W., Weiss, M., Thompson, B., Kashyap, M., Deddens, J., and Pollak, V.E. (1987) *Life Sci. 40,* 2317-2324.
29. Kromhout, D., Bosschieter, E.B., and Coulander, C. (1985) *New Engl. J. Med. 312,* 1205-1209.
30. Weiner, B.H., Ockene, I.S., Levine, P.H., Cuenoud, H.F., Fisher, M., Johnson, B.F., Daoud, A.S., Jarmolych, J., Hosmer, D., Johnson, M.H., Natale, A., Vaudreuil, C., and Hoogasian, J.J. (1986) *New Engl. J. Med. 315,* 841-846.
31. Chang, W-C., Murota, S-I., Nakao, J., and Orimo, H. (1980) *Biochem. Biophys. Acta 620,* 159-166.
32. Panaganamala, R.V., Hanamaiah, B., and Merola, A.J. (1981) *Prost. Med. 6,* 233-235.
33. Lennon, E.A., and Poyser, N.L. (1986) *Prost. Leuk. Med. 25,* 1-15.
34. Chang, W-C., and Tai, H-H. (1984) *Life Sci. 34,* 1269-1280.
35. Chiba, S., Abe, K., Kudo, K., Omata, K., Yasujima, M., Sato, K., Seino, M., Imai, Y., Sato, M., and Yoshinaga, K. (1984) *Prost. Leuk. Med. 16,* 347-358.

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Triacylglycerol Structure of Plant and Fungal Oils Containing γ-Linolenic Acid

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The triacylglycerol stereospecific structure was determined for the major plant oils containing γ-linolenic acid (GLA): evening primrose oil (EPO), black currant oil (BCO), borage oil (BO), and *Mucor javanicus* fungal oil (MJO). It was found that GLA, although not α-linolenic acid, resisted pancreatic lipase hydrolysis. Therefore, the 2-position analysis was determined using phospholipase C-generated 1,2-diacylglycerol and phospholipase A_2-generated lysophosphatidylcholine. GLA was found to be concentrated in the 3-position of EPO and BCO, the 2-position of BO, and the 2- and 3-positions of MJO. In BCO, octadecatetraenoic acid (n-3), also a Δ-6 fatty acid, was distributed similarly to GLA, but α-linolenic acid was found predominantly in the 1-position. Linoleic acid was nearly evenly distributed in all positions of EPO and BCO but was concentrated in the 1-position of BO and the 2-position of MJO. Both palmitic and stearic acids were found predominantly in the 1-position of all of the oils. The results demonstrate similarities and differences in the positional distribution of fatty acids in GLA-containing oils.
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γ-Linolenic acid (GLA) (6,9,12-18:3), the Δ6-desaturase metabolite of linoleic acid (LA), is considered the agent responsible for the apparent effectiveness of evening primrose seed oil (EPO) *(Oenothera biennis)* in the treatment of a variety of diseases (1-3), including premenstrual syndrome and atopic eczema, which may be caused by low or impaired Δ6-desaturase activity (4,5). The occurrence of significant levels of GLA in plant sources is rare and first was reported in 1949 for EPO, which contains 8-10% by weight of GLA (6). More recently, higher levels of GLA have been found in borage seed oil (BO) *(Borago officinalis)* (7), with 24-25% GLA, black currant seed oil (BCO) *(Ribes nigrum)* (8), which is 16-17% GLA, and fungal oils, such as *Mucor javanicus* (MJO) with 16-19% GLA.

Unknown among the GLA-containing oils is the distribution of the GLA and other fatty acids in the triacylglycerol structure. The triacylglycerol structure can influence the metabolism of an oil. It has been shown for peanut oil (9,10) and for synthetic oleoylstearoyltriacylglycerols (11) that the triacylglycerol structure can significantly affect fatty acid absorption, particularly when an unusual fatty acid is present. Hence, we chose to determine and compare the stereospecific structure of the major GLA-containing oils.

MATERIALS AND METHODS

Oils. Evening primrose oil and borage oil were obtained from the Efamol Research Institute (Kentville, Nova Scotia). Glanolin brand (American Health, Orangeburg, NY) black currant oil was purchased at a local store. *Mucor javanicus* fungal oil was obtained from Alcolac, Inc. (Baltimore, MD). No oil contained more than 1% 1,2;2,3-diacylglycerol or more than 1.5% 1,3-diacylglycerol.

Pancreatic lipase hydrolysis. Hydrolysis of the oils by porcine pancreatic lipase (Type II, Sigma Chemical Co., St. Louis, MO) was performed essentially according to the method of Luddy et al. (12). To 4 mg oil, 1.1 ml of buffer containing 1 M tris (hydroxymethyl)-amino methane, pH 8.0, 0.15 M $CaCl_2$ and 0.01% bile salts were added. After addition of 4 mg (64 units) of ether-extracted lipase, the mixture was rapidly stirred in a heating block at 37 C for four min. The reaction products were extracted three times with 3 ml ether, concentrated with a nitrogen stream and separated by thin layer chromatography on unmodified Whatman silica gel K6 plates developed in chloroform/acetone/acetic acid (96:4:0.4, v/v/v). Only trace amounts of acyl migration to 1,3-diacylglycerols could be found on the plates.

Synthesis of phosphatidylcholines. Random diacylglycerols (7-9 mg) were produced by the action of the Grignard reagent, ethyl magnesium bromide (Aldrich Chemical Co., Milwaukee, WI), on 60 mg of triacylglycerol according to the method of Myher and Kuksis (13). After separation of the 1,3- and 1,2;2,3-diacylglycerols by thin layer chromatography on unmodified K6 plates, the 1,2;2,3-diacylglycerols were extracted from the silica gel two times with 6 ml chloroform/methanol (2:1, v/v) and evaporated to dryness. Quantitation of the 1,2;2,3- and 1,3-diacylglycerols by gas chromatography of the methyl esters revealed a nearly theoretical ratio of 2.04:1 (SEM = 0.09, n = 15), respectively, indicating that no significant degree of acyl migration had occurred.

Phosphatidylcholines were prepared by phosphorylation of the 1,2;2,3-diacylglycerols with phosphorus oxychloride (Aldrich Chemical Co.) followed by reaction with choline chloride (13). It was critical that the highly hygroscopic choline chloride crystals were washed thoroughly with acetone or pyridine and the adhering solvent removed by evaporation in a gentle nitrogen stream immediately before use. The 1,2;2,3-phosphatidylcholines were purified by thin layer chromatography on K6 plates developed in chloroform/methanol/water (65:35:4, v/v/v) followed by extraction three times with 5 ml of chloroform/methanol (1:1, v/v). Yields were typically 1-2 mg (20-40%).

Phospholipase A_2 hydrolysis. Nearly complete hydrolysis of the 2-position of 1,2-phosphatidylcholine and no hydrolysis of 2,3-phosphatidylcholine was achieved by incubation of half of the 1,2;2,3-phosphatidylcholines, dissolved in 3 ml ether, with one unit of phospholipase A_2 from bee venom (Sigma Chemical Co.) dissolved in 0.6 ml buffer (0.5 M tris (hydroxymethyl)-amino methane and 2 mM $CaCl_2$, pH 7.5). After rapid shaking at 37 C for 45 min, 0.2 ml of 1 N HCl was added and the reaction products extracted with 6 ml chloroform/methanol (2:1, v/v).

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Abbreviations: BCO, black currant oil; EPO, evening primrose oil; GLA, γ-linolenic acid; LA, linoleic acid; LPC, 1-lysophosphatidylcholine; MJO, *Mucor javanicus* fungal oil.

The 1-lysophosphatidylcholine, free fatty acid, and unreacted 2,3-phosphatidylcholine were separated by thin layer chromatography using chloroform/methanol/water (65:25:4, v/v/v). Lack of hydrolysis of the 2,3-phosphatidylcholine was verified using 2,3-dipalmitoylphosphatidylcholine (Sigma Chemical Co.). The nonspecificity of bee venom phospholipase A_2 for different fatty acids was demonstrated by hydrolysis of the phosphatidylcholines prepared from a mixture of triolein, trilinolein, trigamma-linolenin, and trialpha-linolenin (Nu Chek Prep, Elysian, MN).

Phospholipase C hydrolysis. Hydrolysis of 1,2-phosphatidylcholine to 1,2-diacylglycerol with no hydrolysis of 2,3-phosphatidylcholine was accomplished by incubation of the 1,2;2,3-phosphatidylcholines, dissolved in 3 ml ether, with three units of phospholipase C from *Clostridium welchii* (Type XIV, Sigma Chemical Co.) dissolved in 1 ml buffer (1 M tris (hydroxymethyl)-amino methane and 1 mM $CaCl_2$, pH 7.3). After rapidly shaking for 60 min at 37 C and extraction with 6 ml of chloroform/methanol (2:1, v/v), the 1,2-diacylglycerol was isolated by thin-layer chromatography in hexane/ether/acetic acid (50:50:1, v/v/v). No hydrolysis of 2,3-dipalmitoylphosphatidylcholine could be detected, even after six hr of incubation. The phospholipase C was nonspecific toward the phosphatidylcholines prepared from a mixture of triolein, trilinolein, trigamma-linolenin and trialpha-linolenin.

Fatty acid analysis. Fatty acid methyl esters of all final reaction products and small portions of the intermediate products were prepared by addition of 2–3 ml of benzene/14% BF_3 in methanol (1:1, v/v) to the silica gel scrappings or the evaporates in teflon-lined, screw-cap test tubes followed by heating at 90 C for 30 min or incubating at room temperature overnight. After cooling and addition of water, the methyl esters were extracted with 5 ml of hexane, evaporated to dryness with a nitrogen stream, redissolved in 0.35 ml of octane and analyzed by gas chromatography. The addition of benzene to the silica gel scrappings was essential for quantitative esterification for both phospholipids and glycerolipids.

Fatty acid analyses were performed on a Hewlett-Packard 5880A gas chromatograph equipped with a flame ionization detector. Components were separated on a 30 m × 0.32 mm Supelcowax 10 bonded phase carbowax capillary column (Supelco, Bellefonte, PA) at a split ratio of 30 and a column flow rate of 2.4 ml helium/min. The oven was operated isothermally at 200 C or programmed from 180 C to 230 C, depending on the analysis.

RESULTS

Comparison of methods for determining triacylglycerol structure. A comparison of several methods of determining the stereospecific composition of the triacylglycerols

TABLE 1

Comparison of Methods for Triacylglycerol Stereospecific Analysis of Evening Primrose Oil

		Fatty acid (mol %)				
	Positions	16:0	18:0	18:1	18:2n-6	18:3n-6
1. Triacylglycerol (TG)	All	5.9	1.8	7.5	74.8	9.3
2. Free fatty acid (pancreatic lipase)	1+3	8.1 ± 0.5[a]	2.6 ± 0.1	8.5 ± 0.3	75.4 ± 1.3	4.4 ± 0.7
3. (3TG-MG)/2	1+3	8.5 ± 0.2	2.5 ± 0.2	7.7 ± 0.2	72.7 ± 0.3	7.9 ± 0.3
4. 1,3-Diacylglycerol (1,3-DG) (Grignard reaction)	1+3	7.6 ± 0.3	2.3 ± 0.1	7.7 ± 0.2	72.5 ± 0.9	8.9 ± 0.2
5. 1,2;2,3-Diacylglycerol (pancreatic lipase)	1,2+2,3	4.8 ± 0.3	1.7 ± 0.1	7.1 ± 0.3	71.3 ± 1.8	14.3 ± 2.3
6. 1,2;2,3-Diacylglycerol (1,2;2,3-DG) (Grignard Reaction)	1,2+2,3	4.6 ± 0.3	1.7 ± 0.1	7.5 ± 0.1	75.7 ± 0.6	9.7 ± 0.2
7. 1,2-Diacylglycerol (1,2-DG) (phospholipase C)	1+2	6.0 ± 0.4	3.1 ± 0.9	7.9 ± 0.9	74.5 ± 0.2	7.2 ± 0.3
8. 1-Lysophosphatidylcholine (LPC) (phospholipase A_2)	1	9.6 ± 0.3	4.4 ± 0.1	8.0 ± 0.3	73.3 ± 0.6	3.6 ± 0.2
9. 2(1,2-DG)-4(1,2;2,3-DG) + 3TG	1	11.7 ± 0.6	3.8 ± 2.3	8.5 ± 1.4	70.1 ± 3.6	4.0 ± 1.3
10. 2-Monoacylglycerol (MG) (pancreatic lipase)	2	0.8 ± 0.3	0.4 ± 0.2	7.0 ± 0.1	79.0 ± 0.5	12.1 ± 0.3
11. 4(1,2;2,3-DG)-3TG	2	1.0 ± 0.7	2.1 ± 1.3	7.5 ± 0.5	79.6 ± 2.3	10.8 ± 0.8
12. 2(1,2-DG)-LPC	2	1.8 ± 0.1	1.5 ± 1.4	7.6 ± 0.6	79.0 ± 0.4	10.7 ± 0.3
13. Free fatty acid (phospholipase A_2)	2	1.6 ± 0.4	1.5 ± 0.8	7.3 ± 0.3	77.8 ± 0.8	11.4 ± 0.1
14. 3TG-2(1,2-DG)	3	6.8 ± 0.6	0.3 ± 0.4	7.1 ± 1.1	70.8 ± 0.4	13.5 ± 0.5
15. 2(1,3-DG)-LPC	3	5.7 ± 0.7	0.2 ± 0.1	7.3 ± 0.4	71.4 ± 1.5	14.2 ± 0.6

[a]Values are means ± standard deviations for four to six experiments.

in evening primrose oil is shown in Table 1. Pancreatic lipase initially was employed to generate 2-monoacylglycerols, 1,2;2,3-diacylglycerols, and free fatty acids released from the 1- and 3-positions of the oils. The accuracy of the composition of free fatty acids released from the 1- and 3-positions can be checked in two ways, namely by subtracting the 2-monoacylglycerol composition from the triacylglycerol composition and by the composition of the 1,3-diacylglycerol produced by the Grignard reaction. A comparison of these three methods (items 2–4 of Table 1) reveals good agreement for all fatty acids except for GLA, which is present at only about half the concentration in the lipase-released free fatty acids as in the Grignard-generated 1,3-diacylglycerol or in the calculated 1,3-composition, indicating that GLA resists lipase hydrolysis. Further evidence that GLA resists lipase action is apparent from the 45% higher concentration of GLA in the lipase-released 1,2;2,3-diacylglycerol than in the Grignard-generated 1,2;2,3-diacylglycerol (items 5 and 6 of Table 1). The lipase-released 2-monoacylglycerol appears to be less of a concern because its GLA content was only 10% higher ($P < 0.01$) than that of two other methods used for determining the 2-position composition (items 10–12 of Table 1). Similar levels of discrimination against hydrolysis of GLA-containing triacylglycerols by pancreatic lipase also were observed with BO, BCO and MJO (data not shown).

Interestingly, pancreatic lipase hydrolysis of BCO showed a similar discrimination against octadecatetraenoic acid (6,9,12,15-18:4) but not against α-linolenic acid (9,12,15-18:3). Similar discrimination also was found for tripetroselinin (tri-6-18:1) (data not shown). Apparently, triacylglycerols with a fatty acid containing a double bond in the 6-position are poor substrates for pancreatic lipase. Heimermann et al. (14) have reported that triacylglycerols containing octadecenoic acids of varying double-bond position were poor substrates for pancreatic lipase when the double bond was within seven from the carboxyl-end. Furthermore, marine oil docosahexaenoic acid (4,7,10, 13,16,19-22:6) and especially eicosapentaenoic acid (5,8, 11,14,17-20:5) have been shown to resist pancreatic lipase hydrolysis due to the close proximity of their double bonds to the carboxyl-end and perhaps to steric hindrance (15). GLA also has its double bonds situated unusually close to the carboxyl-end and likewise may be expected to resist pancreatic lipase hydrolysis, as was found. Therefore, free fatty acids and 1,2;2,3-diacylglycerols released by pancreatic lipase cannot be used to determine the stereospecific structure of oils containing GLA and lipase-generated 2-monoacylglycerols should only be used as an approximate check of other methods.

The 1-lysophosphatidylcholine (LPC) released from phosphatidylcholine is considered a reliable representation of the 1-position composition. As a check on the LPC composition, a representative 2-position composition was subtracted from the composition of the 1,2-diacylglycerol released by phospholipase C (items 8 and 9 of Table 1), which closely verified the 1-position analysis.

In addition to the pancreatic lipase-released 2-monoacylglycerol, three other methods were used to determine the composition of the 2-position. These methods were free fatty acid released from the 1,2-phosphatidylcholine by phospholipase A_2, subtracting the triacylglycerol composition from the 1,2;2,3-diacylglycerol composition, and subtracting the LPC composition from the composition of the phospholipase C-released 1,2-diacylglycerol. Items 11–13 of Table 1 indicate close agreement between these methods, especially for the unsaturated fatty acids. The GLA content of the free fatty acids was slightly, but significantly, higher than the GLA content determined by the other two methods. The free fatty acid composition generally is considered inferior to other methods due to acyl migration during the phospholipase A_2 reaction and because of the ease of contamination of the free fatty acid fraction (16). Use of the 1,2;2,3-diacylglycerol composition for determining the 2-position composition is an important verification for oils containing easily oxidized fatty acids such as GLA because it is produced in a single two-min reaction; whereas, the free fatty acids and 1,2-diacylglycerols also require a 16-hr phosphatidylcholine synthesis and a one-hr phospholipase incubation, including three thin layer chromatography separations. However, because any error in the 1,2;2,3-diacylglycerol composition is multiplied by four in the calculation, greater variation usually is found than when using the 1,2-diacylglycerol composition, which is multiplied only by two. Therefore, subtracting the LPC composition from the 1,2-diacylglycerol composition is probably the best way to determine the 2-position composition for oils that resist pancreatic lipase hydrolysis and was the method used in this paper.

The composition of the 3-position was determined either by subtracting the phospholipase C-released 1,2-diacylglycerol from the triacylglycerol composition or by subtracting the LPC composition from the Grignard-generated 1,3-diacylglycerol. Even though 1,3-diacylglycerols generally are considered inferior products for triacylglycerol structure determination, due to acyl migration of the 1,2;2,3-diacylglycerols during the Grignard reaction and subsequent separation, the two methods showed excellent agreement (items 14 and 15 of Table 1).

Comparison of the triacylglycerol structure of GLA-containing oils. Using a single method for determining the composition of each acyl position, the structure of the four GLA-containing oils are compared in Table 2. Several differences and similarities between the oils were found. Very little GLA was found in the 1-position of any of the oils except for MJO. The highest levels of GLA were found in the 3-position of EPO and BCO, the 2-position of BO and the 2- and 3-positions of MJO. In BCO, octadecatetraenoic acid (18:4n-3), like GLA, has its first double bond in the 6-position and has a similar distribution to GLA. However, α-linolenic acid, an isomer of GLA with its first double bond in the 9-position, preferentially is acylated in the 1-position of BCO rather than the 3-position as for GLA. Linoleic acid showed a small preference for the 2-position of EPO and BCO, a larger preference for the 2-position of MJO, a preference for the 1-position of BO and an avoidance of the 3-position of BO and MJO. Oleic acid was distributed evenly among all positions for EPO but accumulated in the 3-position of BO and 2-position of MJO. Stearic acid was excluded from the 3-position of EPO and BCO but was concentrated in the 1-position of all of the oils. Palmitic acid was nearly excluded from the 2-position and most concentrated in the 1-position of all the oils. The long-chain monounsaturates (20:1n-9 and 22:1n-9) of BO and the 24:0 of MJO were concentrated in the 3-position.

TABLE 2

Triacylglycerol Stereospecific Analysis of Oils Containing γ-linolenic Acid (18:3n-6)

Oil	Position[a]	Fatty acid (mol %)						
		16:0	18:0	18:1	18:2n-6	18:3n-6	Other	Other
Evening primrose	All	5.9	1.8	7.5	74.8	9.3		
	1	9.6 ± 0.3[b]	4.4 ± 0.1	8.0 ± 0.3	73.3 ± 0.6	3.6 ± 0.2		
	2	1.8 ± 0.1	1.5 ± 1.4	7.6 ± 0.6	78.9 ± 0.4	10.7 ± 0.3		
	3	6.8 ± 0.6	0.3 ± 0.4	7.1 ± 1.1	74.2 ± 4.8	13.5 ± 0.5		
Black currant	All	6.9	1.3	10.8	46.7	15.9	13.0[c]	2.9[d]
	1	14.2 ± 2.7	4.9 ± 0.7	12.6 ± 1.4	42.7 ± 1.2	4.1 ± 0.7	17.2 ± 0.4	0.7 ± 0.2
	2	2.0 ± 1.0	1.5 ± 1.7	14.1 ± 1.1	53.1 ± 0.9	17.4 ± 1.7	8.1 ± 0.4	2.6 ± 0.5
	3	4.4 ± 1.1	0	5.1 ± 1.1	43.8 ± 1.6	25.8 ± 1.7	14.1 ± 0.9	5.6 ± 0.4
Borage	All	10.7	3.0	15.4	38.1	24.8	4.0[e]	2.2[f]
	1	16.9 ± 1.4	5.3 ± 0.2	13.8 ± 0.4	54.3 ± 0.9	4.0 ± 0.3	3.7 ± 0.4	0.3 ± 0.1
	2	2.3 ± 1.2	1.3 ± 1.2	13.7 ± 1.2	42.5 ± 1.3	40.4 ± 2.2	0.8 ± 0.3	0.4 ± 0.4
	3	13.1 ± 1.4	3.1 ± 0.4	18.7 ± 1.2	17.6 ± 1.6	30.1 ± 1.9	7.7 ± 0.2	5.4 ± 0.1
Mucor javanicus	All	18.6	7.1	39.9	8.9	17.9	1.2[g]	0.6[h]
	1	39.2 ± 1.8	11.5 ± 0.7	24.7 ± 0.6	4.7 ± 0.4	13.3 ± 0.5	1.3 ± 0.4	0.4 ± 0.1
	2	0.2 ± 0.2	0	54.3 ± 1.0	18.5 ± 0.7	19.6 ± 0.7	0.7 ± 0.2	0
	3	18.0 ± 1.7	9.8 ± 0.2	40.5 ± 0.7	3.3 ± 0.6	19.6 ± 0.4	1.4 ± 0.2	1.7 ± 0.2

[a]Position 1, lysophosphatidylcholine; position 2, 1,2-diacylglycerol × 2 minus lysophosphatidylcholine; position 3, triacylglycerol × 3 minus 1,2-diacylglycerol × 2.

[b]Values are means ± standard deviations for four to six experiments.

[c]18:3n-3.

[d]18:4n-3.

[e]20:1n-9.

[f]22:1n-9.

[g]14:0.

[h]24:0.

DISCUSSION

The common fatty acids of most plant oils are symmetrically distributed between the 1- and 3-positions with a preferred distribution of saturated fatty acids in the 1- and 3-positions and of linoleic acid in the 2-position (17). Oils containing ≥C20 fatty acids usually show a preferred distribution of these fatty acids in the 3-position. The GLA-containing oils, however, showed considerable deviation from the norm for the common fatty acids due in part to the asymmetric distribution of GLA. In EPO and BCO where GLA was concentrated in the 3-position, stearic acid was excluded from the 3-position and both stearic and palmitic acids were preferentially located in the 1-position. In BO and MJO, linoleic acid was excluded from the 3-position. Interestingly, the linolenic acid (α plus γ) of human plasma triglycerides is also preferentially esterified in the 3-position while palmitic and stearic acids also are located preferentially in the 1-position (18).

Very little is known about the role of triacylglycerol structure in the metabolic fate of fatty acids. An exception is the atherogenic effect of peanut oils, which contain a high amount of very long chain (C20-24) saturated fatty acids, located exclusively in the 3-position. Randomization of peanut oil distributes all the fatty acids equally and greatly reduces its atherogenic potential (10). Several peanut oils containing the same amounts of the very long chain saturated fatty acids were shown to have significantly different atherogenic potential (9). The main difference between peanut oils of high and low atherogenicity was a high level of linoleic acid and a high ratio of linoleic to oleic acid in the 2-position of the highly atherogenic peanut oil. It is thought that the high level of linoleic acid in the 2-position may allow a higher degree of hydrolysis of peanut oil with a consequent greater absorption of the very long chain saturated fatty acids as 3-monoacylglycerols.

Since GLA may be absorbed poorly due to its resistance to pancreatic lipase, it also may be true with GLA-containing oils as with peanut oils that a high linoleic acid or high linoleic acid to oleic acid ratio in the 2-position may facilitate GLA absorption. Of the GLA-containing oils, evening primrose oil has both the highest linoleic acid and linoleic acid to oleic acid ratio in the 2-position. Therefore, if GLA is poorly absorbed in man it is possible that the GLA in EPO may be more efficiently absorbed than from other known GLA-containing oils.

Finally, as in the case of peanut oils and atherogenesis, it is possible that the variations in triacylglycerol composition that we have observed will lead to differences in biological activities between the various GLA-containing oils.

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REFERENCES

1. Puolakka, J., Makarainen, L., Viinikka, L., and Ylikorkala, O. (1985) *J. Reprod. Med. 30,* 149–153.
2. Pye, J.K., Mansel, R.E., and Hughes, L.E. (1985) *Lancet,* 373–377.
3. Wright, S., and Burton, J.L. (1982) *Lancet,* 1120–1122.
4. Brush, M.G., Watson, S.J., Horrobin, D.F., and Manku, M.S. (1984) *Am. J. Obstet. Gynecol. 150,* 363–366.
5. Manku, M.S., Horrobin, D.F., Morse, N.L., Wright, S., and Burton, J.L. (1984) *Br. J. Dermatology 110,* 643–648.
6. Riley, J.P. (1949) *J. Chem. Soc.,* 2728–2731.
7. Tetenyi, P., Hethelyi, I., Okuda, T., and Imre, S. (1974) *Herba Hung. 13,* 61–71.
8. Traitler, H., Winter, H., Richli, U., and Ingenbleek, Y. (1984) *Lipids 12,* 923–928.
9. Manganaro, F., Myher, J.J., Kuksis, A., and Kritchevsky, D. (1981) *Lipids 16,* 508–517.
10. Myher, J.J., Marai, A., Kuksis, A., and Kritchevsky, D. (1977) *Lipids 12,* 775–785.
11. Mattson, F.H., Nolen, G.A., and Webb, M.R. (1979) *J. Nutr. 109,* 1682–1687.
12. Luddy, F.E., Barford, R.A., Herb, S.F., Magidman, P., and Riemenschneider, W. (1964) *J. Am. Oil Chem. Soc. 41,* 693–696.
13. Myher, J.J., and Kuksis, A. (1979) *Can. J. Biochem. 57,* 117–124.
14. Heimermann, W.H., Holman, R.T., Gordon, D.T., Kowalyshyn, D.E., and Jensen, R.G. (1973) *Lipids 8,* 45–47.
15. Bottino, N.R., Vandenburg, G.A., and Reiser, R. (1967) *Lipids 2,* 489–493.
16. Kuksis, A. (1984) *Lipid Res. Methodol. 10,* 77–131.
17. Breckenridge, W.C. (1978) in *Handbook of Lipid Research* (Kuksis, A., ed.) pp. 197–232, Plenum Press, New York, NY.
18. Parijs, J., DeWeerdt, G.A., Beke, R., and Barbier, F. (1976) *Clin. Chim. Acta 66,* 43–52.

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Dietary Fiber Supplements: Effects on Serum and Liver Lipids and on Liver Phospholipid Composition in Rats

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Rats (6 per group) were fed semipurified diets containing either particulate fibers (alfalfa, 10%; cellulose, 10%; bran, 10%), a soluble ionic fiber (pectin 5%), soluble, nonionic fibers (guar gum, 5%; Metamucil, 10%), a mixed fiber preparation (Fibyrax, 10%, or an insoluble, ionic bile acid-binding resin (cholestyramine, 2%). The control group was fed the unsupplemented diet. The feeding period, during which diet and water were provided ad libitum, was 28 days.

Compared with the control group, serum total cholesterol levels were increased by more than 10% in rats fed alfalfa and decreased by more than 10% in rats fed cellulose, guar gum, Fibyrax and cholestyramine. There were no significant differences in percentage of plasma HDL cholesterol. Serum triglycerides were elevated in the groups fed alfalfa, pectin, guar gum or Fibyrax and reduced in the group fed Metamucil. Plasma phospholipids were elevated in rats fed alfalfa or bran, unaffected in rats fed pectin or Metamucil and reduced in the other groups. Liver total cholesterol was elevated in all groups but those fed wheat bran and cholestyramine. The percentage of liver cholesterol present as ester was elevated in every group except that fed cholestyramine. Liver triglycerides were reduced in rats fed guar gum or Metamucil and elevated in those fed alfalfa. Liver phospholipids were lowered in the group fed cellulose.

Liver phospholipids were fractionated by thin layer chromatography to give phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (Sph), lysophosphatidylcholine (LPC) and phosphatidylinositol plus phosphatidylserine (PI + PS). PC was elevated in all test groups (7–25%); PE levels ranged from 14% below to 0.3% above controls; Sph levels were sharply lower (20–53%) in all groups. LPC and PI + PS levels were close to the control value in all test groups.

The results demonstrate that different dietary fibers can affect liver phospholipid composition. In view of the critical roles of phospholipids in many biological reactions, it will be interesting to survey the influence of dietary fiber on phospholipid spectra of other tissues.
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There have been numerous reports on the effects of various dietary fibers on serum and liver lipids of man and other animal species. Many of the data appear in a recent book (1). The lipids analyzed primarily have been free and esterified cholesterol and triglycerides. On occasion, plasma or liver phospholipids also are assayed. There have been no reports of the effects of dietary fiber on the levels of individual phospholipids. In view of our finding that fibers could bind phospholipids in vitro (2), we thought it would be of interest to determine if dietary fibers could influence the phospholipid spectrum of the liver in vivo. Our findings are the basis of this report.

MATERIALS AND METHODS

Male Wistar rats weighing about 200 g were used in this study. They were randomized into groups of equal average weight (6 rats/group) and maintained at 23 C on a 12-hr light-dark cycle. The rats were fed a basal diet containing (g/100 g): dextrose, 55; casein, 25; corn oil, 14; salt mix, 5; and vitamin mix, 1. Additions of fibers to the diet were made at the expense of the dextrose. The additions were alfalfa (10%) (North Hampton County Farm Bureau, Nazareth, PA); cellulose (10%) (Archer Daniels Midland, Decatur, IL); Fibyrax (10%), a mixed fiber preparation obtained from Farma Foods, Inc., McLean, VA; guar gum (5%) (Freeman Industries, Tuckahoe, NY); 7% methoxylated citrus pectin (5%) (Freeman Industries, Tuckahoe, NY); Metamucil (10%) (G.D. Searle and Co., Skokie, IL) this product contained 50% sugar; and cholestyramine (2%) (Mead-Johnson Co., Evansville, IN). The diets were mixed to our specifications and pelleted by Bio-Serv, Frenchtown, NJ.

The rats were provided their respective diets and water ad libitum for 28 days. Before the end of the study, feces were collected for analysis of fecal steroids and divalent cations. The results of the fecal analyses are reported elsewhere (3). The rats were killed by barbiturate injection and sera and livers taken for further analysis.

Sera were analyzed for total (4) and high density lipoprotein (HDL) cholesterol (5), triglycerides (6) and phospholipids (7). Livers were homogenized in chloroform/methanol (2:1, v/v), the lipid extract was dried over anhydrous Na_2SO_4, and aliquots of the dried extract were used for analysis of free and total cholesterol (8), triglycerides (6) and total phospholipid (7). Individual phospholipids were separated by the thin layer method of Skipski et al. (9) using a solvent mixture consisting of chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v). Phospholipids were visualized using iodine vapor (10) and, after the areas on the plate corresponding to individual compounds had been outlined, the iodine was permitted to sublime from the plate, the silicic acid areas scraped off the plate and the phospholipids eluted and analyzed (7). Data were analyzed by analysis of variance (11).

RESULTS

The data on weight gain are presented elsewhere (3). The control rats gained 121 ± 12 g during the four-wk feeding

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**Deceased.

Abbreviations: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI + PS, phosphatidylinositol plus phosphatidylserine; PS, phosphatidylserine; Sph, sphingomyelin.

period. Rats fed cellulose, wheat bran, Fibyrax or cholestyramine showed similar weight gains, namely, 129 ± 6; 127 ± 5; 123 ± 5; and 134 ± 11 g, respectively. Rats fed the other fibers showed a lesser weight gain (76 ± 0.8 g), which was 41% below the average weight (128 ± 2 g) of the other four groups. Actual weight gains (g ± SEM) were alfalfa, 76 ± 9; pectin, 75 ± 14; guar gum, 74 ± 17; and metamucil, 78 ± 16. Differences in weight gain statistically were significant. The feed efficiency ratios ([weight gain/total food intake] × 100) was highest in rats fed the control or cholestyramine-containing diets (23.7 ± 2.4 and 26.0 ± 2.1, respectively), lowest in those fed Metamucil (12.8 ± 2.6), guar gum (12.9 ± 3.0), and alfalfa (14.0 ± 1.7), and intermediate in those fed pectin (15.9 ± 3.0), wheat bran (18.7 ± 0.7), Fibyrax (19.6 ± 0.8), and cellulose (19.8 ± 0.9).

Table 1 is a summary of the serum lipid data. Serum total cholesterol levels for the entire experiment were significantly different. Compared with the fiber free diet, highest cholesterol levels were observed in rats fed alfalfa, wheat bran or pectin and lowest levels in those fed guar gum or Fibyrax. HDL cholesterol levels (as percentage of total cholesterol) among groups were not significantly different and ranged from 62–82%. Serum triglyceride levels were elevated (compared to control) in rats fed alfalfa, pectin, guar gum or Fibyrax (elevation greater

TABLE 1

Serum Lipids of Rats Fed Fiber Supplements (Mean ± SEM)

Fiber supplement (%)	Cholesterol		Triglycerides (mg/dl)	Phospholipids (mg/dl)
	Total (mg/dl)	HDL-C* (%)		
None	110 ± 13	66.6 ± 2.0	51 ± 4	100 ± 9
Particulate				
Alfalfa (10)	127 ± 7	72.3 ± 5.4	70 ± 4	119 ± 6
Cellulose (10)	95 ± 5	77.0 ± 4.2	58 ± 4	86 ± 3
Wheat bran (10)	121 ± 18	62.4 ± 2.7	53 ± 8	121 ± 14
Soluble, ionic				
Pectin (5)	120 ± 10	77.5 ± 2.0	76 ± 5	91 ± 4
Soluble, nonionic				
Guar gum (5)	85 ± 5	72.8 ± 5.2	77 ± 9	79 ± 3
Metamucil (10)	99 ± 4	64.1 ± 3.9	44 ± 5	89 ± 5
Mixed				
Fibyrax (10)	79 ± 14	82.0 ± 6.9	67 ± 7	82 ± 5
Insoluble, ionic				
Cholestyramine (2)	93 ± 8	79.6 ± 2.3	59 ± 8	84 ± 6
ANOV (p<)	0.05	NS**	0.05	0.01

*HDL-C, high density lipoprotein cholesterol.

**NS, not significant.

TABLE 2

Liver Lipids of Rats Fed Fiber Supplements (Mean ± SEM)

Fiber supplement (%)	Cholesterol		Triglycerides (mg/100 g)	Phospholipids (gm/100 g)
	Total (mg/100 g)	% Ester		
None	450 ± 36	19.6 ± 0.8	332 ± 19	2.06 ± 0.07
Particulate				
Alfalfa (10)	541 ± 31	33.3 ± 1.8	407 ± 39	2.09 ± 0.07
Cellulose (10)	523 ± 31	29.8 ± 2.7	317 ± 31	1.51 ± 0.02
Wheat bran (10)	449 ± 21	26.1 ± 2.0	358 ± 29	1.95 ± 0.07
Soluble, ionic				
Pectin (5)	539 ± 27	29.5 ± 4.6	325 ± 38	2.05 ± 0.08
Soluble, non-ionic				
Guar gum (5)	513 ± 19	27.9 ± 3.2	226 ± 37	2.04 ± 0.13
Metamucil (10)	552 ± 42	23.1 ± 3.0	247 ± 34	1.83 ± 0.10
Mixed				
Fibyrax (10)	616 ± 40	40.9 ± 1.0	561 ± 71	1.96 ± 0.12
Insoluble, ionic				
Cholestyramine (2)	462 ± 16	18.0 ± 3.8	283 ± 30	2.00 ± 0.09
ANOV (p<)	0.05	0.01	0.05	0.05

TABLE 3

Phospholipid Distribution in Livers of Rats Fed Fiber Supplements (Percentage ± SEM)

Fiber supplement (%)	Phospholipid*				
	PC	PE	Sph	LPC	PI + PS
None	42.8 ± 1.6	33.9 ± 0.4	9.3 ± 1.3	6.0 ± 0.4	7.6 ± 0.3
Particulate					
Alfalfa (10)	53.7 ± 0.8	30.1 ± 0.8	4.4 ± 0.3	5.5 ± 0.3	7.2 ± 0.4
Cellulose (10)	46.1 ± 1.5	34.0 ± 0.8	7.2 ± 0.5	6.2 ± 0.4	7.6 ± 0.2
Wheat bran (10)	46.9 ± 1.5	30.4 ± 0.8	7.4 ± 1.7	6.4 ± 0.2	8.9 ± 0.7
Soluble, ionic					
Pectin (5)	53.1 ± 1.8	29.3 ± 1.3	5.7 ± 0.5	5.1 ± 0.4	7.4 ± 0.4
Soluble, non-ionic					
Guar gum (5)	48.3 ± 0.4	30.1 ± 0.7	7.0 ± 0.7	6.5 ± 0.2	8.2 ± 0.4
Metamucil (10)	47.4 ± 1.9	31.8 ± 0.8	6.9 ± 0.6	6.9 ± 0.4	7.9 ± 0.3
Mixed					
Fibyrax (10)	46.4 ± 1.6	31.6 ± 0.7	5.5 ± 0.2	7.5 ± 0.5	9.1 ± 0.3
Insoluble, ionic					
Cholestyramine (2)	47.5 ± 0.6	33.8 ± 0.8	5.5 ± 0.7	5.9 ± 0.3	7.9 ± 0.5
ANOV (p<)	0.01	0.01	0.05	0.01	0.05

*PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphinogmyelin; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine.

than 30%). Triglyceride levels in the other groups were within the normal range. Serum phospholipids were highest in the rats fed alfalfa or wheat bran and lowest in those fed Fibyrax or cholestyramine. Rats fed alfalfa or wheat bran appeared to have the highest levels of total serum lipids.

Liver lipids (Table 2) were significantly different among groups. Total liver cholesterol was highest in rats fed Fibyrax or Metamucil. The percentage of cholesterol present as ester was within the normal range only in the rats fed cholestyramine or Metamucil (normal being defined here as control value ± 15%). Liver cholesterol levels were lowest in the groups fed the soluble, nonionic fibers and highest in the group fed Fibyrax. Liver phospholipid levels were considerably below normal levels only in rats fed cellulose.

The distribution of individual liver phospholipids is summarized in Table 3. Differences among groups are statistically significant for each phospholipid. Levels of PC were elevated in all test groups with the differences ranging from 7 to 25%. PE levels were equal to or slightly lower than the control value in all test groups. (Control, 33.9 ± 0.4%; average for all test groups, 31.4 ± 0.6%.) Sph content was reduced considerably in all the test groups with reductions ranging from 20 to 53%. Levels of LPC and of PI + PS generally were close to control levels in all groups. The range of difference of LPC was from −15% (pectin) to +25% (Fibyrax); the range for PI + PS was from −5% (alfalfa) to +20% (Fibyrax) but with control levels of LPC and PI + PS being 6.0 ± 0.4 and 7.6 ± 0.3%, the averages of the test groups were 6.3 ± 0.3 for LPS and 8.0 ± 0.2 for PI ± PS.

DISCUSSION

In this study, we compared simultaneously the effects of many types of dietary fiber: particulate, soluble ionic and nonionic, and mixed. No distinct or consistent patterns of effects on serum or liver lipids emerge. Generally, rats fed alfalfa exhibited the highest overall levels of serum lipids. If we arbitrarily assume that levels of lipids 15% higher or lower than those seen in the control group are indicative of an effect, we find lowering of serum cholesterol by guar gum and Fibyrax; elevation of serum triglycerides in rats fed alfalfa, pectin, guar gum or Fibyrax; and elevation of serum phospholipids in rats fed alfalfa or wheat bran and reduction in those fed guar gum. Liver total cholesterol levels were elevated in rats fed every fiber, but wheat bran, guar gum and cholestyramine and esterified cholesterol was elevated in every group except that fed cholestyramine. Triglycerides were elevated in rats fed alfalfa or Fibyrax and reduced in those fed guar gum or Metamucil. Liver phospholipid levels were reduced only in rats fed cellulose.

Although the values for total cholesterol are different, direct comparison showed no significant differences (by Student's t-test) between any of the test groups and the control. However, rats fed cellulose, Metamucil, Fibyrax or cholestyramine all excreted twice as much total bile acid (8.8 ± 0.19 mg/day) as did the control group (4.4 ± 0.7 mg/day) (3). Compared directly with the control group, only rats fed alfalfa or pectin exhibited significantly elevated triglyceride levels. These differences cannot be explained on the basis of weight gain or feed efficiency.

Total liver cholesterol levels cannot be correlated with either fecal bile acid or neutral steroid excretion. The level of hepatic cholesteryl ester content is an indication of cholesterol deposition. The finding that hepatic cholesteryl ester levels were 18–113% higher than control in every group except that fed cholestyramine was surprising and suggests an effect on endogenous cholesterol metabolism that merits further study.

In examining the distribution of liver phospholipids, two consistent effects were observed. Liver PC was higher

than the control level in all fiber-fed groups, and liver Sph consistently was lower in all test groups. The average percentages of PC and Sph in livers of control rats were 42.8 ± 1.6 and 9.3 ± 1.3%, respectively. Average PC and Sph for the eight test groups were 48.7 ± 1.1 and 6.2 ± 0.4. The ranges of PE, LPC and PI + PS content were small and within a few percentages of the control values.

To our knowledge, this is the first study of the effects of dietary fiber on liver phospholipid spectrum. The observed differences suggest that dietary fibers may play a hitherto unsuspected role in membrane structure and in lipid metabolism in general. It would be of future interest to determine if fiber affected the phospholipid composition of specific lipoprotein molecules.

The influence of dietary fibers on phospholipid metabolism also may be of interest with regard to tumor growth. There are differences between membrane phospholipids of rat or mouse liver and rat or mouse hepatomas (12,13). Comparison of phospholipid of normal and transformed human diploid cells showed two significant differences; PS levels were lower in the transformed cells and Sph levels were higher. Levels of all other phospholipids were unchanged in the transformed cells (14).

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REFERENCES

1. Spiller, G.A. (ed.) *Handbook of Dietary Fiber in Human Nutrition* (1986), CRC Press Inc., Boca Raton, FL.
2. Vahouny, G.V., Tombes, R., Cassidy, M.M., Kritchevsky, D. and Gallo, L.L. (1980) *Lipids 21,* 1012-1018.
3. Vahouny, G.V., Khalafi, R., Satchithanandam, S., Watkins, D.W., Story, J.A., Cassidy, M.M., and Kritchevsky, D. (1987) *J. Nutr. 17,* 2009-2015.
4. Zlatkis, A. and Zak, B. (1969) *Anal. Biochem. 29,* 143-148.
5. Lipid Research Clinics (1974) *Lipid and Lipoprotein Analysis,* Vol. 1, DHEW Publication No. NIH 75-638, Bethesda, MD.
6. Levy, A.L., and Keyloun, C. (1972) *Adv. Automated Analysis 1,* 487-502.
7. Sokoloff, L., and Rothblat, G.H. (1974) *Proc. Soc. Exp. Biol. Med. 146,* 1166-1172.
8. Sperry, W.M., and Webb, M. (1950) *J. Biol. Chem. 187,* 97-106.
9. Skipski, V.P., Peterson, R.F., and Barclay, M. (1964) *Biochem. J. 90,* 374-378.
10. Kritchevsky, D., and Kirk, M.R. (1952) *Arch. Biochem. Biophys. 35,* 346-351.
11. Sokal, R.R., and Rohlf, F.J. (1969) *Introduction to Biostatistics,* W.H. Freeman and Co., San Francisco, CA.
12. Bergelson, L.D., and Dyatlovitskaya, E.V. (1973) in *Tumor Lipids: Biochemistry and Metabolism* (Wood, R., ed.), pp. 111-125, American Oil Chemists' Society, Champaign, IL.
13. van Hoeven, R.P., and Emmelot, P. (1973) in *Tumor Lipids: Biochemistry and Metabolism* (Wood, R., ed.), pp. 126-138, American Oil Chemists' Society, Champaign, IL.
14. Howard, B.V., and Kritchevsky, D. (1969) *Int. J. Cancer 4,* 393-402.

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Relative Inhibitory Potencies of Flavonoids on 12-Lipoxygenase of Fish Gill

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The relative efficacy of 10 flavonoid compounds and some common antioxidants in inhibiting 12-lipoxygenase of fish gill was determined. Lipoxygenase activity was measured by oxygen consumption with polarography and formation of hydroxy fatty acid using thin layer chromatography with autoradiography. Generally, the inhibition of 12-lipoxygenase by flavonoids was noncompetitive. The lipoxygenase product pattern did not change in the presence of flavonoids. Fisetin (3,3′,4′,7-tetrahydroxyflavone) and quercetin (3,3′,4′,5,7-pentahydroxyflavone) were the most potent inhibitors of 12-lipoxygenase, with IC_{50}'s of 0.25 and 0.4 μM respectively. These were compared with IC_{50}'s values of 5, 1000 μM for BHA (t-butylhydroxyanisole) and BHT (t-butylhydroxytoluene) respectively. Possible inhibitory mechanisms and relationships between flavonoid structure and inhibitory potencies are discussed.
Lipids 23, 322–326 (1988).

The high degree of unsaturation of the component polyunsaturated fatty acids renders fish lipids very susceptible to oxidation. Though lipid oxidation is thermodynamically favored, direct reaction between unsaturated fatty acids and oxygen is spin-forbidden (1). This kinetic spin restriction can be overcome by free radical reactions, but these require an activation mechanism to facilitate the initiation reaction to produce catalytic free radicals (1). Several enzymatic and nonenzymatic processes such as autoxidation photosensitized oxidation, lipoxygenase, peroxidase and microsomal enzymes have been suggested to generate reactive oxygen species that can function as initiators of lipid oxidation in muscle tissues (1–4). Because all of these intiation mechanisms may occur, their relative importance and individual contribution to the overall processes needs to be understood. The ability of lipoxygenase to produce fatty acid hydroperoxides and its widespread occurrence in animal tissues suggest that it may be important in initiation of lipid oxidation (2,5,6).

Peroxy-enzyme radical complexes exist during the catalytic cycle of lipoxygenase (7,8). These release hydroperoxides that serve as sources of free radicals. Currently, it is not clear if lipoxygenase functions similarly to free radicals as an initiator of lipid oxidation. In this regard, specific inhibitory compounds could be useful in differentiating between lipoxygenase reactions and other free radical reactions. Compounds with free radical quenching ability should inhibit free radical oxidation but may not be lipoxygenase inhibitors. Flavonoids, with their free radical quenching and metal chelating abilities, are potential inhibitors of lipid oxidation (9–11). The available evidence indicates that some flavonoids may specifically interfere with the lipoxygenase reactions and their inhibitory potencies may be related to their structures rather than their free radical quenching abilities (12–17). Thus, it is possible that two positional isomers of hydroxyl groups of flavonoids may have similar free radical quenching abilities but differ in their capacity to inhibit lipoxygenase. This suggests that structurally different flavonoids may be used as specific inhibitors of lipoxygenase; thereby helping to distinguish lipoxygenase from other free radical reactions.

In this report, the highly active 12-lipoxygenase of gill tissue (2,5) was used in a model system to study the inhibition of lipid oxidation by various hydroxy isomers of flavonoids and some common antioxidants such as nordihydroguaiaretic acid (NDGA), propyl gallate (PG), t-butylhydroxyanisole (BHA) and t-butylhydroxytolune (BHT). The differing inhibitory effects of these compounds on lipoxygenase are demonstrated and discussed.

MATERIALS AND METHODS

Materials. Arachidonic acid (20:4,n-6) was obtained from NuChek Prep (Elysian, MN). [1-^{14}C]arachidonic acid was bought from New England Nuclear (Boston, MA). Caffeic acid, esculetin, glutathione (GSH), BHA, BHT, PG, NDGA and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). Eicosatetraynoic acid (ETYA) was a gift from W. Scott (Hoffman LaRoche, Nutley, NJ). Flavonoids such as fisetin, apigenin, naringenin, catechin, 3-epicatechin, chrysin, myricetin, quercetin, rutin and esculin were obtained from G. Hrazdina (Institute of Food Science, Cornell University). Ethyl acetate was obtained from Fisher Scientific Co. (Rochester, NY). Methanol was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Chloroform and glacial acetic acid were from Mallinckrodt Inc. (St. Louis, MO).

Lipoxygenase preparation. Samples of gill tissue (1 g) carefully were excised from young, freshly killed rainbow trout (15 g) (Tunison Fish Laboratory, Cortland, NY) and homogenized in 40 ml 0.05 M (pH 7.4) phosphate buffer with 1 mM glutathione using a Polytron homogenizer. The homogenate was centrifuged for 15 min. at 15,000 × g at 4 C. The resultant supernatant fraction was used as the crude enzyme source without further purification (2,5,6). The enzyme preparation either was used immediately or stored at −70 C as beads of droplets instantaneously frozen in liquid nitrogen. Protein concentration was estimated by phenol reagents with bovine serum albumin as standard (18).

Lipoxygenase assay. To verify the activity and specificity of the lipoxygenase reactions and to determine inhibitory effects of flavonoids, the enzyme preparation (1 mg protein/ml) was incubated at 25 C with arachidonic acid (50 μM) using labeled [1-^{14}C]arachidonic acid as tracer. The extent of enzymatic reaction was calculated from the conversion of radioactive substrate fatty acid into its hydroxy analog following 10 min incubation. The reaction was quenched by acidification with formic acid

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Abbreviations: BHA, t-butylhydroxyanisole; BHT, t-butylhydroxytolune; ETYA, eicosatetrahynoic acid; GSH, glutathione; NDGA, nordihydroguaiaretic acid; PG, propyl gallate; TLC, thin layer chromatography.

to pH 3.0 and the products were extracted twice with 1 ml ethyl acetate as described (2). Extracted lipids were separated by thin layer chromatography (TLC) using a solvent system consisting of chloroform/methanol/acetic acid/water (90:8:1:0.8, v/v/v/v) (19). The radiolabeled product, 12-hydroxy-5,8,10,14-eicosatetraenoic acid, from labeled arachidonic acid, was generated due to the activity of gill lipoxygenase and was detected qualitatively by autoradiography as reported (20). The extent of product formation during the reaction period was quantified by locating, and scraping the corresponding bands and measuring by liquid scintillation counting the radioactivity in each band (5,6).

Calculations of enzyme velocity. The rate of lipoxygenase-catalyzed reaction was determined by oxygen consumption using a polarographic cell (Hansatech oxygen electrode, Hansatech Ltd., Norwich, England). For enzyme assays, the lipoxygenase preparation (1 mg protein/ml) was equilibrated in phosphate buffer (0.05 M, pH 7.4) in a water-jacketed oxygen electrode chamber at 25 C for one min. Then the flavonoid or specific inhibitor in ethanol (1 μl) was introduced into the enzyme assay mixture and incubated for 10 min. The enzyme activity then was initiated by the addition of arachidonic acid (25 nmol) in ethanol (1 μl), and the oxygen concentration was monitored by the electrode and recorded continuously using a microcomputer (Fig. 1a). The experimental data were smoothed to eliminate random electronic noise (21). The first differential of the oxygen concentration vs time data (dO_2/dt) was calculated (Fig. 1b). The maximal rate and lag time required to reach this rate were obtained from these first differential plots. The appropriate programs for performing these analyses were written in Forth for the Apple IIe.

Relative inhibitory efficacy of flavonoids and antioxidants on gill lipoxygenase. Various antioxidants, and flavonoids were tested to compare their inhibitory effects on gill lipoxygenase. The gill lipoxygenase was prepared as described above. The enzyme was preincubated with an inhibitor at 25 C for 10 min. The radiolabeled substrate, arachidonic acid, then was added to initiate enzyme reaction. The reaction was stopped after an additional 10 min. incubation; the products were separated by thin layer chromatography and detected by autoradiography as mentioned above. The inhibitory effect of each inhibitor was estimated by measuring the radioactivity in the product by liquid scintillation counting as stated above. At least five concentrations of each inhibitor were employed to determine its inhibitory potency. The IC_{50} value, the concentration of inhibitor causing 50% reduction of enzyme activity, was calculated by linear regression analysis (22).

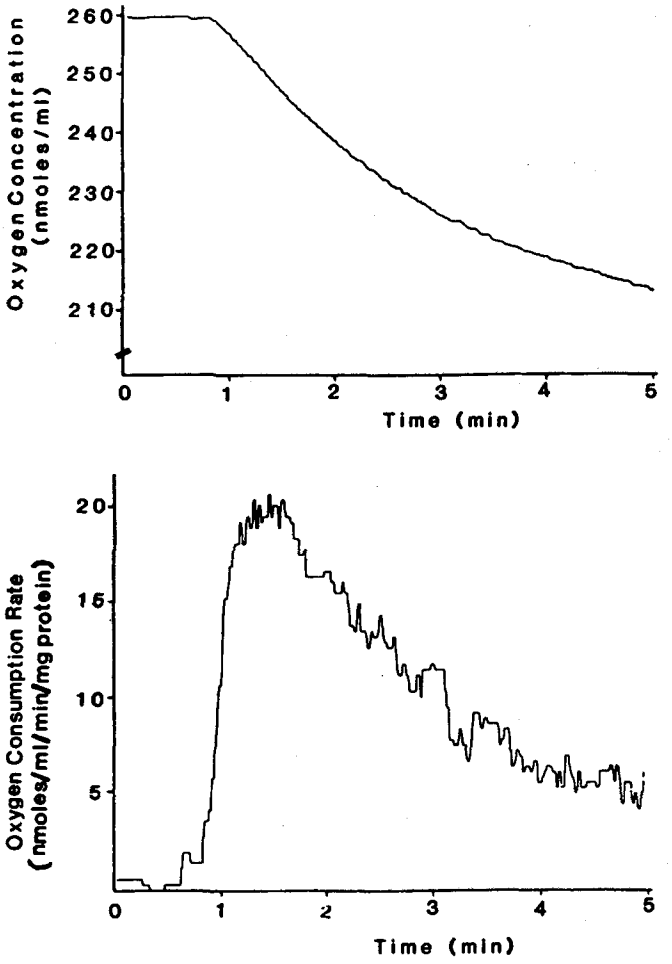


FIG. 1. The progress curve (1a) and the first differential (1b) of oxygen consumption by 12-lipoxygenase-catalyzed oxidation of arachidonic acid. Gill 12-lipoxygenase (0.5 mg protein) in 0.5 ml phosphate buffer was incubated at 25 C for one min. Oxygen consumption following the addition of fatty acid was measured by polarography. Reaction details as described in Materials and Methods.

RESULTS

A significant and practical advantage of using trout gill lipoxygenase is its freedom from other oxygen-consuming reactions (20). The rate of lipoxygenase-catalyzed reaction was determined from oxygen consumption. The addition of arachidonic acid to the gill enzyme system resulted in rapid oxygen consumption after a brief lag phase (Fig. 1a and 1b). The formation of product was accompanied by the consumption of oxygen as shown on Fig. 1a. The apparent Km-value of gill 12-lipoxygenase for arachidonic acid as determined by polarography was 20 μM (Fig. 2).

The specificity of lipoxygenase reaction was verified by TLC analysis of the products formed following incubation with radiolabeled arachidonic acid (peak I) (Fig. 3), which showed that the main reaction product (peak II) was 12-hydroxy-5,8,10,14-eicosatetraenoic acid (2,5). The addition of inhibitors reduced product formation but did not change the nature of the product.

Effect of enzyme inhibitors. The effects of various inhibitors on product formation were determined using thin layer autoradiography and liquid scintillation counting of TLC products bands. Indomethacin (1–100 μM), a known cyclooxygenase inhibitor, exerted no inhibitory effect on the gill lipoxygenase, indicating the absence of cyclooxygenase in the gill preparation. The nonspecific lipoxygenase competitive inhibitor, ETYA, inhibited gill enzyme activities (IC_{50} = 50 μM) (Table 1). Esculetin caused a dose-dependent inhibition on gill lipoxygenase system with an IC_{50} of 0.02 μM (Table 1).

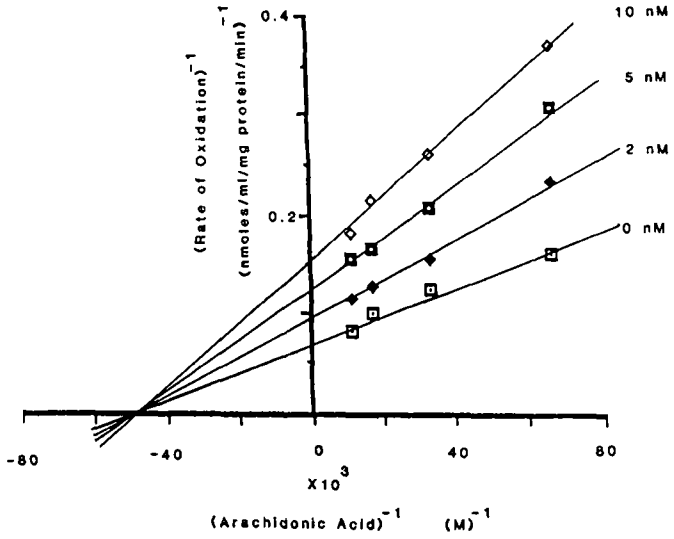


FIG. 2. Lineweaver-Burk plot showing the inhibition of trout gill 12-lipoxygenase by fisetin. Gill 12-lipoxygenase (0.5 mg protein) in 0.5 ml phosphate buffer was incubated at 25 C for one min. Various concentrations of fisetin were added to the enzyme assay system and incubated for an additional 10 min. Oxygen consumption, following the addition of arachidonic acid, was measured by polarography. Reaction details as described in Materials and Methods.

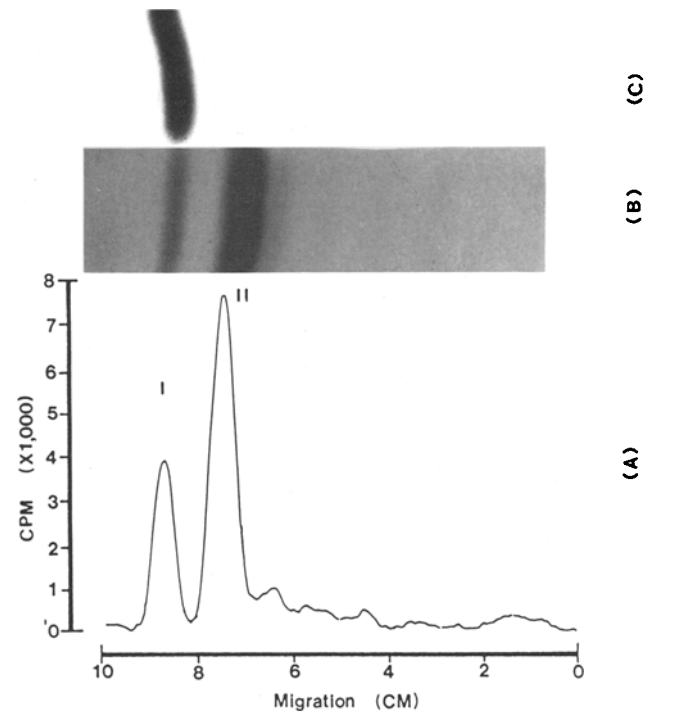


FIG. 3. Autoradiogram and its scan (a) of products generated during 10 min incubation of arachidonic acid (50 μM) with the 12-lipoxygenase from trout gill in the absence (b) and presence (c) of 10 μM fisetin. Extraction, TLC and detection of labeled metabolites were performed as described in Materials and Methods. Peak I is arachidonic acid, 30% of the total area; whereas, peak II is its 12-hydroxy fatty acid, 70% of the total area.

Various antioxidants and free radical quenchers were tested to compare their inhibitory effects. Propyl gallate was a potent inhibitor of gill lipoxygenase (IC_{50} = 0.02 μM) while NDGA was less effective (IC_{50} = 2.5 μM). The widely used phenolic antioxidant BHA also inhibited gill 12-lipoxygenase moderately (IC_{50} = 5 μM), whereas BHT, a similar type of antioxidant, had negligible inhibitory effects (IC_{50} > mM). Comparable disparities among BHA, BHT, PG and NDGA in inhibitory potencies previously were observed for lipoxygenase of mice epidermis (23) and reticulocytes (7).

The inhibitory effects of specific hydroxy isomers of flavonoids on lipoxygenase revealed that fisetin and quercetin were the most potent of all the flavonoids tested with IC_{50}'s of 0.25 and 0.4 μM, respectively (Table 1). These compounds possess a catechol (1,2-benzenediol) structure in the B ring (3′ = hydroxyl, 4′ = hydroxyl). Whereas chrysin (3′ = hydrogen, 4′ = hydrogen) (IC_{50} = 30 μM), naringenin (3′ = hydrogen, 4′ = hydroxyl) (IC_{50} = 40 μM), and apigenin (3′ = hydrogen, 4′ = hydroxyl) (IC_{50} = 5 μM) were 100, 100 and 10 times less inhibitory, respectively. Hydroxylation at the 5′-carbon of quercetin decreased the inhibitory effect as exemplified by myricetin (IC_{50} = 2 μM). Hydrogenation of the double bond at the 2-carbon position and absence of the carbonyl function at the 4-carbon position reduced the inhibitory efficiency as exemplified by catechin (IC_{50} = 3 μM). 3-epicatechin (IC_{50} = 1 μM) was almost as active as catechin as an inhibitor of gill lipoxygenase (Table 1). Glycosidation at 3-hydroxyl group, such as the addition of rutinose (6-o-a-L-rhamnosyl-D-glucose) to quercetin to yield rutin (IC_{50} = 7 μM) or the addition of glucose (6-glucoside) to esculetin (IC_{50} = 0.02 μM) to give esculin (IC_{50} = 500 μM) resulted in loss of the inhibitory potency of these compounds (Table 1).

A dihydroxyl group on flavonoids was required for inhibition of rat 5-lipoxygenase (16) and 15-lipoxygenase (13,16). The position and number of hydroxyl groups in the coumarin skeleton are important for the inhibition of platelet 12-lipoxygenase (15) and the 5-lipoxygenase in polymorphonuclear leukocytes (24).

The mechanism of the inhibitory effects of flavonoids on 12-lipoxygenase activity was assayed by polarography at various concentrations of arachidonic acid in the presence of increasing concentrations of inhibitors. The data for fisetin showed a concentration-dependent inhibition of lipoxygenase in the nanomolar range, and the inhibition was consistant with noncompetitive mechanism. The IC_{50} value of fisetin on lipoxygenase obtained by polarography was consistent with that determined by TLC-radioactivity counting. The product specificity was not altered in the presence of inhibitors.

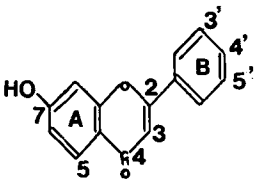
Similar noncompetitive inhibition of 12-lipoxygenase of rat platelet; 5-lipoxygenase of mast tumor cells; and 5-, and 12-lipoxygenase of rat basophilic leukemia cells have been reported for esculetin (15), caffeic acid (25), and cirsiliol(3′,4′,5′-trihydroxy-6,7-dimethoxyflavon) (26), respectively.

DISCUSSION

Phenolics, catechols and pyrogallols, with oxidation potential 0.92, 0.74 and 0.61 volts, respectively, traditionally are considered as antioxidants that scavenge

TABLE 1

The Relative Inhibitory Effects of Various Phenols, Flavonoids, and Antioxidants on Fish Gill 12-Lipoxygenase



Test compounds	Substituents					IC_{50} (μM)[a]	Radical quenching[b]	Oxidation potential[c] (volts)
	3-C	5-C	3'-C	4'-C	5'-C			
Chrysin	H	OH	H	H	H	30	0	—
Apigenin	H	OH	H	OH	H	5	0.40	—
Fisetin	OH	H	OH	OH	H	0.25	0.68	—
Quercetin	OH	OH	OH	OH	H	0.40	9.94	—
Myricetin	OH	OH	OH	OH	OH	2	—	—
Rutin	rutinose	OH	OH	OH	H	7	0.92	—
Catechin[d]	OH	OH	OH	OH	H	3	0.72	—
Epicatechin[d]	OH	OH	OH	OH	H	1	0.76	—
Naringenin[e]	H	OH	H	OH	H	40	0.28	—
Esculetin						0.02	—	0.76
Esculin						500	—	—
Caffeic acid						20	—	—
ETYA						50	—	—
BHA						5	—	0.65
BHT						1000	—	0.68
PG						0.02	—	—
NDGA						2.5	—	—

[a]Reactions were performed in the standard radioactive and polarographic assays mixture containing 1 mg partially purified enzyme. The enzyme was preincubated with inhibitor at 25 C for 10 min. Then the substrate fatty acid was added for reaction.

[b]Data derived from ref. 13. 0 denotes no quenching effect toward the free radical generated by human erythrocytes incubated with phenazine methosulphate, while 1 corresponds to 100% quenching effect.

[c](27,32).

[d]Flavan.

[e]Flavanone.

scavenge alkoxy and alkperoxy radicals (27,28). The effects of BHA, BHT (phenolics), caffeic acid, NDGA (catechols), and PG (pyrogallol) indicated that the inhibition of gill lipoxygenase by these antioxidants was not related to their oxidation potentials. BHA and BHT have similar oxidation potentials (0.65 and 0.68 volts respectively), but BHA was a much stronger lipoxygenase inhibitor than BHT. NDGA, with a catechol structure, has a lower oxidation potential than BHA (27). Nonetheless, NDGA showed the same inhibition potency as BHA. Caffeic acid with a catechol structure was a weaker lipoxygenase inhibitor than NDGA. PG (with a pyrogallol structure) had a lower oxidation potential than esculetin (with a catechol structure) (27), but they showed similar inhibition potencies toward lipoxygenase. These findings suggest that the oxidation potential of an antioxidant is not related to its inhibitory potency on lipoxygenase. Comparable disparities in inhibitory potencies among BHA, BHT and NDGA were reported in reticulocyte and mice skin lipoxygenases (7,23). Schewe et al. attributed the difference in inhibition to the structure and accessibility of antioxidants toward the catalytic site in the lipoxygenase in which an enzyme radical complex is formed (7).

Flavonoids by virtue of their free radical quenching, electron transport and metal chelating abilities are potential inhibitors of lipid oxidation (9–11). Specific requirements for the number and position of hydroxy groups, as exemplified by catechol (1,2-benzenediol) structure in the B rings of quercetin and fisetin, for the inhibition of lipoxygenase-initiated lipid oxidation were indicated from the data. Catechol, with an oxidation potential of 0.74 volts, is easily oxidized and capable of scavenging superoxide and hydroxy radicals and inhibiting lipid oxidation (29,30). Replacement of the hydroxy group in the catechol structure (B ring) with hydrogen (e.g. apigenin, with a phenol structure, and chrysin, with a benzene structure) resulted in higher oxidation potential, higher radical quenching ability (27) but lower inhibitory potencies on lipoxygenase. Myricetin, with a pyrogallol structure, has a lower free radical quenching ability and was a less potent lipoxygenase inhibitor than quercetin.

Esculetin, with a catechol structure and oxidation potential 0.76 volts (31), is a potent inhibitor of fish lipoxygenase. Glycosidation at one of the hydroxy groups of catechol structure converts esculetin to esculin that is a less potent lipoxygenase inhibitor than esculetin.

However, the presence of catechol structure is not sufficient for flavonoids to be lipoxygenase inhibitors. Glycosidation of a hydroxy group such as the 6-glycosidation of quercetin to rutin, change neither the catechol structure nor the free radical quenching ability (14). But rutin is 20 times less potent than quercetin as a lipoxygenase inhibitor. Alternation of the flavonoid structure from a flavone (such as quercetin) to a flavan (such as catechin and epicatechin), or from a flavone (such as apigenin) to a flavanone (such as naringenin) retains the catechol structure in the B ring and has similar free radical quenching abilities (14). Nonetheless, catechin and epicatechin are less potent than quercetin as 12-lipoxygenase inhibitors. Naringenin is not as potent as apigenin in inhibiting lipoxygenase activity. These findings indicate that small alternations in the flavonoid structure that did not influence oxidation potential or free radical quenching ability, markedly changed the inhibition potency of 12-lipoxygenase.

Molecules with a catechol structure can form complexes with the active ferric form of soybean and reticulocyte lipoxygenases and thereby cause inhibition (7,32). This implies that the small structural change such as glycosidation may influence the conformation of flavonoid and hinder its accessibility to the active site of lipoxygenase and lower its inhibitory potency.

Our observations suggest that both oxidation potential and specific structure should be considered in choosing flavonoids as selective lipoxygenase inhibitors. Particular pairs of flavonoids with minor differences in their structures and similar free radical quenching ability, e.g. esculetin and esculin or quercetin and rutin, may be used as selective lipoxygenase inhibitors to distinguish lipoxygenase reactions from other free radical involved reactions. The fish gill lipoxygenase system, because of its high lipoxygenase and absence of cyclooxygenase activities, may be useful for studying the metabolism of lipoxygenase pathways.

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REFERENCES

1. Frankel, E.N. (1984) *Prog. Lipid Res. 22,* 1-33.
2. German, J.B., and Kinsella, J.E. (1986) *Biochim. Biophys. Acta 875,* 12-20.
3. Kanner, J., and Kinsella, J.E. (1983) *J. Agri. Fd. Chem. 31,* 370-376.
4. Slabyj, B.M., and Hultin, H.O. (1984) *J. Fd. Sci. 49,* 1392-1393.
5. German, J.B., and Kinsella, J.E. (1985) *J. Agri. Fd. Chem. 33,* 680-683.
6. Hsieh, R.J., and Kinsella, J.E. (1986) *J. Fd. Sci. 51,* 940-945.
7. Schewe, T., Rapoport, S.M., and Kuhn, H. (1986) *Adv. Enzymol. 58,* 191-272.
8. Vliegnthart, J.F.G., and Veldink, G.A. (1980) in *Free Radicals in Biology* (Fryer, W.A., ed.), pp. 29-41, Academic Press, New York.
9. Havsteen, B. (1983) *Biochem. Pharmcol. 32,* 114181148.
10. Baumann, J., Bruchhausen, F.V., and Wurm, G. (1982) in *Flavonoids and Bioflavonoids,* 1981 (Farkas, L., Gabor, M., Kallay, F., and Wagner, H., eds.), pp. 411-419, Elsevier Sci. Publ. Co., New York.
11. Pratt, D.E. (1976) in *Phenolic, Sulfur, and Nitrogen Compounds in Food Flavors* (Charalambous, G., ed.), ACS Symposium Series No. 26, pp. 1-13, Washington, D.C.
12. Wauwe, J.V., and Goossens, J. (1983) *Prostaglandins 26,* 725-730.
13. Baumann, J., Bruchhausen, F.V., and Wurm, G. (1980) *Prostaglandins 20,* 627-639.
14. Baumann, J., Wurm, G., and Bruchhausen, F.V. (1980) *Arch. Pharm. 313,* 330-337.
15. Sekiya, K., Okuda, H., and Arichi, S. (1982) *Biochim. Biophys. Acta 713,* 68-72.
16. Yoshimoto, T., Furukawa, M., Yamamoto, S., Horie, T., and Watanabe-Kohno, S. (1983) *Biochem. Biophys. Res. Commun. 116,* 612-618.
17. Welton, A.F., Tobias, L.D., Fiedler-Nagy, C., Anderson, W., Hope, W., Meyers, K., and Coffey, J.W. (1986) in *Plant Flavonoids in Biology and Medicine: Biochem., Pharmacol, and Structure-Activity Relationships* (Cody, V., Middleton Jr., E., Harborne, J.B., eds.), pp. 231-242, A.R. Liss, Inc., New York.
18. Lowry, O.H., Farr, A.L., Rosenbrough, N.J., and Randall, R. (1951) *J. Biol. Chem. 193,* 265-275.
19. Powell, W.S. (1982) *Methods Enzymol. 85,* 467-477.
20. German, J.B., and Kinsella, J.E. (1986) *Biochim. Biophys. Acta 879,* 378-387.
21. Tukey, J.W. (1977) *Exploratory Data Analysis,* Addison Wesley Publ., Reading, MA.
22. Steel, R.G.D., and Torrie, J.H. (1980) in *Principles and Procedures of Statistics: A Biometrical Approach,* 2nd edn., pp. 239-271, McGraw-Hill Book Co., New York.
23. Nakadate, T., Yamamoto, S., Aiza, E., and Kato, R. (1984) *Gann 75,* 214-222.
24. Kimura, Y., Okuda, H., Arichi, S., Baba, K., and Kozawa, M. (1985) *Biochim. Biophys. Acta 834,* 224-229.
25. Koshihara, Y., Neichi, T., Murota, S., Lao, A., Fujimoto, Y., and Tatsuno, T. (1984) *Biochim. Biophys. Acta 792,* 92-97.
26. Kohno, S.W., Ohata, K., Maki, Y., Horie, T., Yoshimoto, T., and Yamamoto, S. (1985) *Adv. Prostaglandin Thromboxane Leukotriene Res. 15,* 217-219.
27. Scott, G. (1965) in *Atmospheric Oxidation and Antioxidants* (Scott, G., ed.), pp. 115-169, Elsevier Pub. Co., New York.
28. Sherwin, E.R. (1972) *J. Am. Oil Chem. Soc. 49,* 418-422.
29. Pincemail, J., Deby, C., Lion, Y., Braquet, P., Hans, P., Drieu, K., and Goutier, R. (1986) in *Flavonoids and Bioflavonoids, 1985* (Farkas, L., Gabor, M., and Kallay, F., eds.), pp. 423-436, Elsevier Pub. Co., New York.
30. Hodnick, W.F., Roettger, W.J., Kung, F.S., Bohmont, C.W., Pardin, R.S. (1986) in *Plant Flavonoids in Biology and Medicine: Biochem., Pharmacol., and Structure-Activity Relationships* (Cody, V., Middleton, E., Harborne, J.B., eds.), pp. 249-252, A.R. Liss, Inc., New York.
31. Fieser, L.F. (1930) *J. Am. Chem. Soc. 52,* 5204-5240.
32. Spaapen, L.J.M., Verhagen, J., Veldink, J., and Vliegenhart, J.F.G. (1980) *Biochim. Biophys. Acta 617,* 132-140.

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Changes in Plasma Lipids, Lipoproteins, Triglyceride Secretion and Removal in Chicks with Estrogen Implants

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Estradiol implants in chicks resulted in marked elevation of all major plasma lipids with greatest increase in triglyceride (TG) followed by phospholipid (PL) and cholesterol (C). During the two-wk period, plasma TG level in estrogen (E)-treated chicks increased to about 45 times that of controls (139.6 vs 6,368.3 mg/dl). The level of cholesterol also increased steadily during the same period, attaining nearly a six-fold increase in comparison with the control (150.7 vs 871.8 mg/dl), and the level of PL was markedly elevated from 209 to 2,861 mg/dl. Besides the induction of hyperlipidemia, E treatment also resulted in a notable alteration in the fatty acid composition of plasma lipids; there was an increase in oleic acid concomitant with a decrease in polyunsaturated fatty acids, particularly, linoleic acid. One day after implantation, the percentage of oleic acid in TG fraction increased from 39.2 to 43.7%, reaching 55.4% of the total fatty acids at day 14. In contrast, the levels of linoleic and arachidonic acid decreased significantly from 16.1 to 8.3% and 4.3 to 0.6%, respectively, during the same period. In cholesteryl ester (CE) and PL, the oleic acid level also increased from 25.2 to 47.3% in the former and from 11.9 to 29.6% in the latter, reflecting enhanced hepatic lipogenesis. Analysis of plasma lipoproteins in E-treated chicks revealed dramatic alterations in the concentrations of lipids and protein in individual lipoprotein fractions, especially very low density lipoprotein (VLDL) fraction. The respective levels of TG, C and PL in the VLDL fractions were 10.0, 0.6 and 1.0 mg/dl in the control, and 3,904.4, 530.3 and 1,365.2 mg/dl in chicks implanted with estrogen for seven days. The concentrations of TG, C and PL also were markedly increased in the low density lipoprotein (LDL) fraction in these birds. However, the cholesterol content of the high density lipoprotein (HDL) fraction was decreased dramatically in E-treated chicks (47.1) relative to the control (121.5 mg/dl). The protein level in the VLDL fraction from E-treated chicks was profoundly elevated to a level 300-fold greater than controls. TG secretion rates were measured in vivo following the administration of Triton WR-1339. In control chicks, plasma TG secretion rate was 2.29 mg/min; whereas, chicks treated with E for one and three days showed significantly higher TG secretion rates of 3.18 and 5.27 mg/min, respectively. TG removal rates were measured in vivo after administration of a 10% fat emulsion. Although plasma TG concentrations were different between control and E-treated birds, no significant differences were found in TG removal rates in those birds, indicating no impairment of TG clearance in E-treated chicks.
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Abbreviations: C, cholesterol; CE, cholesterol ester; CHD, coronary heart disease; E, estrogen; EDTA, ethylenediamine tetraacetic acid; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; PL, phospholipid; TG, triglyceride; VLDL, very low density lipoprotein.

Epidemiological evidence that women have a lower incidence of coronary heart disease (CHD) than age-matched men has led to the popular theory that endogenous estrogenic hormones in some way protect women against atherosclerosis (1). This suspected protective effect coupled with reports of reduced coronary atherosclerosis with exogenous estrogen treatment in some experimental animals (2,3) stimulated the testing of estrogens for the prevention of CHD in man. However, human trials in subjects with prior myocardial infarction resulted in an increased rather than decreased risk of thromboembolism (4). Similarly, the use of exogeneous estrogen in the treatment of advanced prostatic cancer resulted in increased arterial and venous thromboembolism and mortality (5). In addition, an elevation of the concentration of plasma TG was a frequent metabolic side-effect induced by the estrogenic component of oral contraceptives (6,7). The widespread use of such preparations, and the well-established association between hyperlipidemia and CHD has generated some interest in the mechanism underlying estrogen-induced hyperlipidemia (8,9). However, studies of human endogenous hypertriglyceridemia have provided conflicting accounts; the results of some kinetic studies indicate increased production (9–11), while others indicate diminished disposal of TG (12,13).

In birds, estrogen administration causes hyperlipidemia (14), a phenomenon that is spontaneously developed in hens upon sexual maturity for the egg laying. Estrogen-induced hyperlipidemia is characterized by the marked elevation in the levels of VLDL and, to the less extent, LDL, but a concomitant decrease in HDL (15). The HDL-lowering effect of estrogen in birds appears to be opposite from that of humans in whom HDL generally increases (16). However, the development of hyperlipidemia in both humans and avian species as a consequence of estrogen treatment establishes the chick as a suitable animal model to study TG metabolism.

The present study, therefore, was undertaken to examine changes in the levels of plasma lipids (triglyceride [TG], cholesterol [C] and phospholipid [PL]) and the composition of lipoproteins (very low density lipoprotein [VLDL], low density lipoprotein [LDL] and high density lipoprotein [HDL]) in chicks following estrogen implants. Since estrogen-induced hyperlipidemia could be due to overproduction or underdisposal, the TG secretion and its removal rates were investigated in vivo using Triton WR-1339 and Intralipid, respectively. Because of possible changes in the fatty acid profiles under estrogenic influence, the fatty acid composition of plasma lipids in these chicks also was determined.

MATERIALS AND METHODS

Animals. Male crossbred 10–13-wk-old chicks (New Hampshire male × Colombian female) were used in these experiments. These birds had wing veins large enough to

facilitate blood withdrawal. The birds were housed individually in wire cages under a consistent lighting program (09:00-21:00 hr). Chicks were fed ad libitum a regular grain diet (University of Illinois pretest chick diet No. 4) composed of 23% crude protein, 3% fat, 5% crude fiber, over 60% carbohydrate and a mineral and vitamin premix. The fatty acid composition of the grain diet was as follows (%): myristic—0.8; palmitic—15.9; palmitoleic—0.8; stearic—3.0; oleic—30.6; linoleic—47.6 and linolenic—1.3.

Estrogen implants. For the study of time-course changes in individual plasma lipids under estrogenic influence, 10 birds were randomly selected and implanted with estrogen-filled tubes. Estrogen tubes were prepared by the method of Smith et al. (17). Briefly, polymethylsiloxane tubing (Silastic, Dow Corning Corp., Midland, MI; 1.5 mm i.d. × 3.13 mm o.d.) was cut into 6-cm length, filled with ca. 50 mg of β-estradiol dipropionate powder (Sigma Chemical Co., St. Louis, MO), and both ends of tubes were sealed with Medical Adhesive Silicon Type A. Before implantation, the tubes were incubated in 0.01 M phosphate-buffered saline solution (pH 7.4) for 48 hr at 37 C. Under light ether anesthesia, the neck skin of chicks was incised (0.5-1.0 cm) with a surgical knife, and tubes were inserted through the incisions. Incisions were closed with an Auto Suture skin stapler. Empty tubes constructed in the same way were implanted into control chicks.

Triglyceride secretion. The in vivo secretion of TG in chicks was determined following Triton WR-1339 (p-isooctyl polyoxyethylene phenol polymer) injection (18). In preliminary experiments, series of Triton concentration were administered into estrogen-treated hyperlipidemic chicks (10-wk-old) to determine the dosage of Triton, which adequately will block the TG removal. Chicks with plasma TG values ranged from 1,527 to 2,925 mg/dl were administered 200, 400, 500, 600, 700 and 800 mg Triton/kg body weight via wing vein. Blood samples were taken at 60 min after the Triton injection and plasma TG contents were determined. The doses of 600 mg or above of Triton resulted in maximum accumulation of TG (Fig. 1), indicating an adequate inhibition of TG removal at these Triton concentrations. In the main experiments, 12 male chicks (10-wk-old) fed the basal grain diet were selected randomly. Eight chicks were implanted with estrogen-filled tubes, and the rest were used as sham-operated controls. In the estrogen group, the TG secretion study was carried out one and three days after implantation and in the control group, two days after sham operation. The chicks were fasted overnight (14 hr), and Triton WR-1339 was injected intravenously into the right wing vein at the level of 600 mg/kg body weight. Blood samples were taken from the left wing vein at 0, 30 and 60 min after the Triton injection. The TG secretion rate was calculated by multiplying the slope of the least-squares regression of plasma TG concentration over time with the plasma volume. The plasma volume was estimated by the total blood volume based on body weight and hematocrit measurement (19).

Triglyceride removal. The in vivo TG removal rate was determined following the IV injection of a 10% fat emulsion (Intralipid, Cutter Laboratories, Inc., Berkeley, CA) as described by Carlson and Rossner (20). Twelve male chicks (13-wk-old) maintained on a grain diet were selected randomly. Eight chicks were implanted with estrogen-filled tubes, and the rest were used as sham-operated controls. TG removal rate was measured one and three days after the implantation in the estrogen group and two days after the sham operation in the control group, respectively. The chicks were fasted overnight (14 hr), and Intralipid (10% emulsified lipid solution) was injected intravenously into the right wing vein at the level of 500 mg/kg body weight. Blood samples were collected from the left wing vein at 0, 10, 20 and 30 min after the Intralipid injection. The TG removal rates were calculated by multiplying the slope of the least-squares regression of plasma TG concentration over time, with the plasma volume estimated by the total blood volume based on body weight and hematocrit measurement (19).

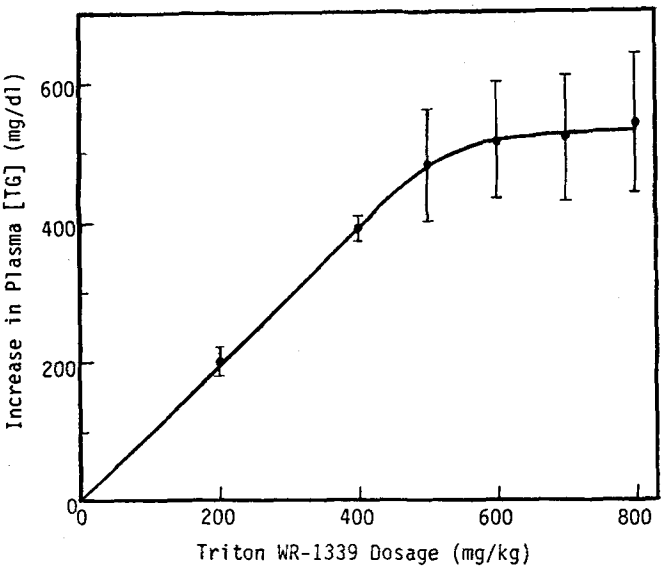

FIG. 1. Effects of various Triton WR-1339 concentrations on the plasma triglyceride accumulation in estrogen-treated chicks. Each point of the curve represents the mean ±SD values of three animals.

Lipoprotein analysis. Chicks implanted with estrogen for seven days and control chicks that were fasted overnight had blood samples drawn from the wing vein into tubes containing ethylenediamine tetraacetic acid (EDTA) (1.0 mg/ml blood). The separation of plasma lipoproteins was carried out by sequential preperative ultracentrifugation with Ti 60 rotor at 12 C in a Beckman Model L5-50 ultracentrifuge as described by Havel et al. (21). Three lipoprotein fractions were isolated: VLDL ($\varrho <$ 1.006 g/ml), LDL (1.006 $< \varrho <$ 1.063 g/ml) and HDL (1.063 $< \varrho <$ 1.21 g/ml). Within each lipoprotein fraction, the total C, TG and PL contents were analyzed using the methods described for plasma lipids below. The protein content within each lipoprotein fractions was determined by the method of Lowry et al. (22).

Agarose gel electrophoresis. The whole plasma and isolated lipoproteins from the control and E-treated chicks were electrophoresed in 0.6% agarose gels with 0.5% BSA in 0.05 M barbital buffer at pH 8.6 as described by Noble (23). Gels were stained for lipid with Sudan Black B.

Analysis of blood lipids and fatty acid composition. After an overnight fast, blood samples were drawn from the wing vein of the control and E-implanted chicks into

tubes containing EDTA (1 mg/ml of blood) by means of butterfly needles (Abbott Hospital, Inc., Chicago, IL). The plasma was separated by centrifugation at 1,500 × g for 20 min at 4 C. The level of plasma total cholesterol was determined enzymatically (24) by using A-Gent cholesterol reagent (Abbott Laboratories, Inc., Chicago, IL). The value of plasma TG was measured by the method of Foster and Dunn (25). For the determination of plasma PL content, total lipid material was extracted from an aliquot of plasma according to the method of Folch et al. (26), and then the total PL value was determined from aliquots of lipid extracts after perchloric acid digestion (27). Aliquots of lipid extracts from blood plasma also were applied to silica gel thin layer plates, and neutral lipids were separated by petroleum ether/diethyl ether/acetic acid (85:15:1, v/v/v). Lipid spots were detected by spraying rhodamine 6G (0.025% in 95% ethanol) and viewing under ultraviolet light. For the analysis of fatty acid composition, the areas on thin layer plates corresponding to CE, TG and PL were scraped into glass ampules, and fatty acid methyl esters were prepared in the presence of silica gel with 4% (v/v) sulfuric acid in absolute methanol at 90 C for two hr. The analyses of fatty acid methyl esters were carried out with a Hewlett-Packard 5710A gas chromatograph as described (28).

Data were analyzed by the difference between means, and statistical significance was based on Student's t-test or Fisher's Least Significant Difference multiple comparison test (29).

RESULTS

Time-course changes in plasma TG, C and PL levels in chicks following estrogen treatment are shown in Table 1. The implantation of estradiol resulted in marked elevation of all major plasma lipids, with the greatest increase in TG followed by PL and C. One day after implantation, the TG level increased almost three-fold, and this pattern of increase progressed almost linearly until day 14. During the two-wk period, plasma TG levels in estrogenized birds increased to about 45 times that of the controls (139.6 vs 6,368.3 mg/dl). Although the elevation of C was not as dramatic as that of TG, the level of C increased steadily during the same period, attaining nearly a six-fold increase in comparison with the controls (150.7 vs 871.8 mg/dl). As the level of plasma total C increased, the percentage of free C rose from 37.9% to 57.1%. During the two-wk treatment, the level of plasma PL also increased significantly from 209.0 to 2,861.4 mg/dl. During the 14-day period, in untreated birds no changes in those plasma lipid levels were observed.

Changes in the fatty acid composition of plasma TG during estrogen treatment are shown in Table 2. In control chicks, oleic acid was the major fatty acid (39.2%) of plasma TG followed by palmitic (25.5%), linoleic (16.1%) and stearic (10.7%) acid; a low level of arachidonic acid also was present (4.3%). Estrogen treatment resulted in substantial alteration of the fatty acid composition with a marked increase in a monoenoic fatty acid and concomitant decreases in polyunsaturated fatty acids. One day after implantation, the percentage of oleic acid increased from 39.2% to 43.7%; this increase progressed steadily with time, reaching about 55% of the total fatty acids by day 14. In contrast, the level of linoleic acid decreased significantly from 16.1% to 8.3%, and the arachidonic acid level also decreased from 4.3% to 0.6% during the same period. Changes in the saturated fatty acids were less obvious, and both palmitic and stearic acids decreased only slightly by day 14.

TABLE 1

Time-course Changes of Plasma Lipids in Chicks Implanted with Estrogen[a]

Day	Triglyceride	Cholesterol	Phospholipid
		Control	
0	141.0 ± 25.2	146.6 ± 10.5	206.1 ± 11.6
2	134.2 ± 20.4	144.2 ± 13.0	200.8 ± 20.3
14	139.6 ± 26.1	150.7 ± 14.8	180.6 ± 33.4
		Estrogen-treated	
0	140.1 ± 30.7	149.7 ± 14.5 (37.9)[b]	209.0 ± 28.2
1	431.6 ± 68.1	—	—
2	1,248.0 ± 387.6	246.1 ± 42.6 (40.9)	620.4 ± 56.7
3	1,378.0 ± 317.6	—	—
4	—	365.1 ± 52.1 (46.3)	923.4 ± 95.3
5	2,287.4 ± 178.1	—	—
6	—	482.6 ± 37.9 (51.8)	1,409.3 ± 62.5
14	6,368.3 ± 521.8	871.8 ± 131.2 (57.1)	2,861.4 ± 356.0

[a]Mean (mg/dl) ± S.D. Control chicks, n = 5; estrogen-treated chicks, n = 10.

[b]Percentage (%) of free cholesterol.

TABLE 2

Time-course Changes in Fatty Acid Compositions of Plasma Triglyceride, Cholesteryl Ester and Phospholipid in Chicks Implanted with Estrogen

	Fatty acids (%)[a]						
Days	14:0[b]	16:0	16:1	18:0	18:1	18:2	20:4
				Triglyceride			
0	1.2	25.5	3.0	10.7	39.2	16.1	4.3
1	0.6	29.3	4.8	9.3	43.7	11.2	1.1
3	0.5	27.8	5.2	7.9	48.4	9.7	0.5
5	0.4	26.7	4.3	7.2	50.3	10.4	0.7
14	0.5	23.7	3.5	8.0	55.4	8.3	0.6
				Cholesteryl ester			
0	0.6	17.6	4.1	3.3	25.2	45.4	3.8
1	0.7	20.2	3.8	6.5	26.7	42.4	2.7
3	1.2	19.0	5.3	3.1	35.3	34.2	1.9
5	0.4	19.1	4.2	3.2	41.6	30.0	1.5
14	1.7	19.4	4.5	4.1	47.3	21.5	1.5
				Phospholipid			
0	0.3	27.4	0.8	26.0	11.9	14.7	18.9
1	0.2	31.1	1.2	22.2	14.6	14.7	16.0
3	0.2	31.0	1.3	20.2	21.9	13.9	11.5
5	0.2	30.7	1.3	17.8	26.1	12.9	11.0
14	0.3	30.7	1.1	18.2	29.6	9.9	10.2

[a]Average values of triplicate analyses of pooled samples from 10 birds.

[b]Number of carbon atoms:number of double bonds.

In normal chicks, plasma CE contained a very high level of unsaturated fatty acids, which composed nearly 80% of the total fatty acids (Table 2). Among fatty acids esterified to C, linoleic acid was predominant (45.4%), followed by oleic (25.2%) and palmitic acid (17.6%). As was the case in TG, the estrogen treatment resulted in a significant increase in oleic acid from 25.2% to 47.3%, whereas linoleic acid decreased from 45.4% to 21.5% during the 14-day period. The arachidonic acid level decreased slightly, but the levels of saturated fatty acids were relatively unchanged. The fatty acid composition of plasma PL differed from that of other plasma lipid classes because of its high level of arachidonic acid, which composed about 19% of the total fatty acids (Table 2). The level of saturated fatty acid also was very high, with palmitic and stearic acids composing 53% of the total fatty acids. As in the cases of plasma TG and CE, estrogen treatment also resulted in notable changes in the fatty acid composition of phospholipid, especially with regard to oleic acid. During the 14-day period, the level of oleic acid increased from 11.9% to 29.6%. During the same period, the level of stearic, linoleic and arachidonic acid decreased from 26.0% to 18.2%, 14.7% to 9.9%, and 18.9% to 10.2%, respectively.

The whole plasma and isolated lipoproteins were electrophoresed on the agarose gel (Fig. 2). In control chicks, the VLDL, LDL and HDL had three distinct electrophoretic mobilities (pre-β, β, α), whereas the lipoprotein of pre β-mobility was absent in E-treated chicks. In contrast with the control VLDL, the VLDL from E-treated chicks exhibited β-mobility similar to LDL, which may indicate that VLDL and LDL from E-treated chicks are indistinguishable. In fact, the presence of a continuum of particles in the VLDL-intermediate density lipoprotein (IDL)-LDL range; the similarity in the apoprotein composition of VLDL and LDL from E-treated chicks (15) supports this possibility.

Table 3 shows the distribution of TG, C, PL and protein in the VLDL, LDL and HDL fractions of the control and chicks implanted with estrogen for seven days. From the total lipid values shown, the control chicks were normolipidemic, while E-treated chicks were grossly hyperlipidemic, and the hyperlipidemia was reflected primarily in the VLDL-TG. Compared with the control, VLDL-TG increased 386-fold in E-treated chicks (10.1 vs 3,904.4 mg/dl). For LDL-TG, the increase was about eight-fold, but HDL-TG concentration remained the same as that of the control. Concerning the distribution of C, the VLDL-C showed an 829-fold increase in E-treated chicks, relative to the value in the control. For LDL-C, there was a 1.4-fold increase, but HDL-C showed a 2.6-fold decrease in the E-treated chicks, relative to the control values. For phospholipid, there was an overwhelming 1,379-fold increase in the VLDL of E-treated chicks compared with the control values. The LDL-PL in the E-treated chicks was 3.4-fold that in the control. However, compared with the value in the control, HDL-PL showed a 1.7-fold decrease in E-treated chicks. The protein concentration in the VLDL fraction of E-treated chicks was elevated profoundly. Relative to control values, the increase was about 300-fold. LDL-protein was increased about three-fold, but HDL-protein showed a 2.7-fold decrease in E-treated chicks relative to the values in the controls.

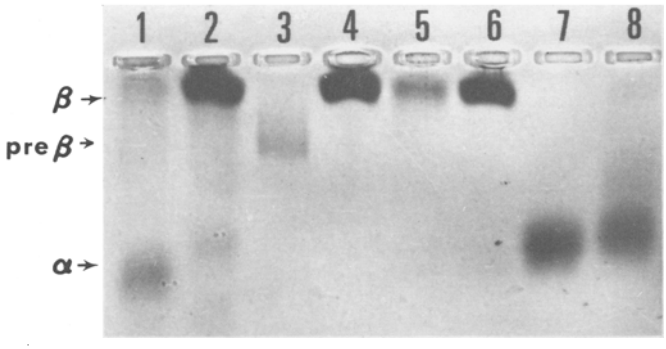

FIG. 2. Agarose gel electrophoresis of whole plasma and isolated lipoproteins from control (C) and estrogen-treated (E) chicks: (1) C-plasma, (2) E-plasma, (3) C-VLDL, (4) E-VLDL, (5) C-LDL, (6) E-LDL, (7) C-HDL, (8) E-HDL.

TG secretion rates in control and esterogen-treated chicks was monitored in vivo following the administration of Triton WR-1339 as shown in Table 4. The action of Triton upon TG metabolism is thought to be mediated by its detergent properties that bind TG, thereby inhibiting lipoprotein lipase activity (18). The linear increases in plasma TG concentrations were obtained in both control and estrogen-implanted chicks during the 60-min post-Triton period. In the control chicks, the level of plasma TG increased from the initial level of 140.6 to 257.0 mg/dl, resulting in a net TG secretion rate of 2.29 mg/min. Chicks implanted with estrogen for one day had an initial TG level of 388.7 mg/dl, which increased to 547.4 mg/dl at 60 min after Triton WR-1339 treatment. The average TG secretion rate in those birds was 3.18 mg/min, and was significantly higher than that of control chicks. Chicks implanted with estrogen for three days had a plasma TG level of 1,048.6 mg/dl that rose to 1,305.9 mg/dl at 60 min after Triton treatment, resulting in the TG secretion rate of 5.27 mg/min. The measurement of TG secretion rate in chicks with estrogen implant for more than five days was not possible due to the development of extreme hypertriglyceridemia in those birds.

TG removal was measured in control and estrogen treated chicks following Intralipid (10% fat emulsion) administration as shown in Table 5. The concentrations of plasma TG decreased linearly in both control and estrogen-treated chicks during the 30 min post-Intralipid period. In control chicks, the plasma TG level at 10 min after the Intralipid injection was 697.6 mg/dl, which decreased to 409.9 mg/dl at 30 min, resulting in a TG removal rate of 15.34 mg/min. In chicks with estrogen implants for one and three days, their plasma TG levels reached 827.5 and 1,195.2 mg/dl at 10 min and decreased to 574.5 and 941.9 mg/dl at 30 min after Intralipid injection, resulting in TG removal rates of 13.81 and 13.58 mg/min, respectively. In spite of marked differences in plasma TG levels in those birds, no significant differences were found in TG removal rates between control and estrogen-treated chicks.

DISCUSSION

Hypertriglyceridemia associated with estrogen administration has been reported in humans (6–9) and animals (14,30). In rats, estrogen treatment produces a biphasic effect; high doses decrease plasma cholesterol, and lower

TABLE 3

Composition of Plasma Lipoproteins in Chicks with Estrogen Implants[a]

	Triglyceride	Cholesterol	Phospholipid	Protein
Control				
Plasma	114.41 ± 11.29	159.55 ± 18.23	216.32 ± 19.73	449.73 ± 54.43
VLDL	10.11 ± 2.24	0.64 ± 0.12	0.99 ± 0.22	2.86 ± 0.20
LDL	24.56 ± 1.79	37.42 ± 7.06	31.53 ± 3.73	33.13 ± 4.03
HDL	79.75 ± 11.08	121.50 ± 16.5	183.80 ± 18.56	413.75 ± 54.79
Estrogen-treated[b]				
Plasma	4180.42 ± 639.41	629.94 ± 87.77	1578.11 ± 255.23	1113.75 ± 152.26
VLDL	3904.37 ± 636.36	530.26 ± 95.67	1365.23 ± 268.25	856.25 ± 162.82
LDL	202.86 ± 27.36	52.55 ± 10.52	106.50 ± 18.61	104.00 ± 19.73
HDL	73.19 ± 1.87	47.14 ± 8.38	106.38 ± 13.86	153.50 ± 19.69

[a]Values are means (mg/dl) ± SD (n = 6). Chicks were implanted with estrogen for seven days.

[b]All the values of estrogen-treated group, except HDL triglyceride, are significantly different from the corresponding values of control group ($p < 0.01$).

TABLE 4

Effects of Estrogen Treatment in Chicks on Triglyceride Secretion Rate[a]

	Plasma TG (mg/dl)[b]			TG secretion rate (mg/min)
	0 time	30 min	60 min	
Control	140.6 ± 11.3[a]	195.0 ± 17.2[a]	257.0 ± 28.4[a]	2.29 ± 0.52[a]
Estrogen				
Day 1	388.7 ± 76.7[b]	471.7 ± 73.3[b]	547.4 ± 52.0[b]	3.18 ± 0.75[b]
Day 3	1,048.6 ± 381.7[c]	1,184.4 ± 380.6[c]	1,305.9 ± 369.1[c]	5.27 ± 0.72[c]

[a]The body weights of control and estrogen-treated chickens were 1,962 ± 130 g and 1,991 ± 168 g, respectively.

[b]Mean ± S.D. (n = 4). Means with different superscripts in the same column are significantly different ($p < 0.05$).

TABLE 5

Effects of Estrogen Treatment in Chicks on Triglyceride Removal Rate[a]

	Plasma TG (mg/dl)[b]				TG removal rate (mg/min)
	0 time	10 min	20 min	30 min	
Control	161.3 ± 6.7[a]	697.6 ± 190.7	541.4 ± 165.5	409.9 ± 138.9	15.34 ± 6.71[a]
Estrogen					
Day 1	340.5 ± 25.0[b]	827.5 ± 50.1	737.0 ± 29.7	574.5 ± 59.8	13.81 ± 3.29[a]
Day 3	841.9 ± 25.5[c]	1,195.2 ± 132.1	1,031.7 ± 69.8	941.9 ± 50.6	13.58 ± 3.98[a]

[a]The body weights of control and estrogen-treated chickens were 1,880 ± 250 g and 1,805 ± 137 g, respectively.

[b]Mean ± S.D. (n = 4). Means with different superscripts in the same column are significantly different ($p < 0.01$).

doses elevate it (30). In this study, the implantation of β-estradiol dipropionate into chicks resulted in a marked elevation of plasma lipids, and particularly TG, in which a three-fold increase was noted within 24 hr. Elevation of the plasma TG level progressed almost linearly during the 14 days of treatment. Besides hypertriglyceridemia, estrogen treatment in chicks also induced hypercholesterolemia and hyperphospholipidemia. The degree of increases in cholesterol and phospholipid was much less than that in triglyceride, but the time-course changes of these lipids exhibited a similar pattern of elevation during the 14-day period. Since the chicks were fed a low-fat, cholesterol-free grain diet, it is very clear that these plasma lipids were from endogenous synthesis.

In addition to elevating plasma lipids, estrogen treatment altered the fatty acid composition of TG, CE and

PL. Estrogen implantation induced a significant modification of fatty acid composition in triglyceride as well as phospholipid and cholesteryl ester. Among the fatty acids, the level of oleic acid was most affected. The increase in the level of oleic acid was accompanied by a decrease in the level of polyunsaturated fatty acids such as linoleic and arachidonic acids. This finding is similar to that observed in hens with increased lipogenesis at the onset of sexual maturity (31). The selective increase in the level of oleic acid indicates that both de novo fatty acid synthesis and fatty acid desaturation are stimulated greatly under estrogenic influence. Several studies have shown a strong correlation between increased hepatic lipogenesis in animals treated with estrogen and hyperlipidemia (14,32). The activities of the hepatic lipogenic enzymes, fatty acid synthetase and acetyl-CoA carboxylase were reported to be increased in chicks (33) and rats (34) following estrogen treatment. Estrogen also has been shown to affect gene transcription, resulting in an increased hepatic production of mRNA for apo VLDL-II, an apoprotein of VLDL (35). Therefore, estrogen seems to have the concerted effect of increased hepatic lipogenesis and secretion of triglyceride-rich VLDL.

Present data on the lipoprotein composition revealed remarkable increases in the concentrations of TG, C, PL and protein in VLDL and, to lesser extent, in LDL fractions, but these lipoprotein components were found to be decreased significantly in the HDL fraction with E treatment. These findings are consistent with those reported by Kudzma et al. (15). The factor underlying the decrease in HDL production is not clear. However, recent findings in estrogenized chicks and turkeys (15,36) suggest estrogen may cause an adverse effect on the production of apoprotein A-1, a major lipoprotein of HDL. Since the synthesis of vitellogenin and VLDL are stimulated markedly under estrogenic influence, reduced synthesis of other plasma proteins such as apoprotein A-1 may be the result of competition at the level of mRNA translation on the endoplasmic reticulum (36). There appears to be an opposite effect of estrogen on HDL between mammals and avian species. Although estrogen treatment in birds lowers the level of HDL in plasma (15), there are reports that in humans (16) and rats (37) treated with estrogen the level of HDL is increased. However, in light of the inverse relationship between HDL concentration and the incidence of coronary heart disease (38) this influence of estrogen on the modulation of HDL production needs to be elucidated.

Beside compositional changes in lipoproteins, the distribution of lipoprotein particles also appears to be modified in estrogen-induced hyperlipidemia. Kudzma et al. reported the presence of a continuum of particles of the VLDL-IDL-LDL range in E-treated chicks, whereas lipoproteins of density less than 1.06 g/ml were clearly separated into discrete VLDL and LDL fractions in untreated birds (15). This observation seems to indicate that conventional density intervals applied for the isolation of plasma lipoproteins may not reveal sufficient details about discrete lipoprotein subspecies in E-treated hyperlipidemic chicks; the use of continuous density gradient ultracentrifugation can provide more information regarding the multiple subspecies.

In this study, the hepatic TG secretion rate observed in vivo following the administration of Triton WR-1339 strongly indicates that an accelerated secretion of hepatic triglyceride into the circulation plays an important role in the endogeneous hypertriglyceridemia of estrogenized chicks. Compared with the control, the treatment with estrogen resulted in a significantly higher rate of triglyceride secretion, which was much greater after three days than after one day. Similar findings have been reported in rats in which hepatic triglyceride secretion increased following the injection of estradiol benzoate for 21 days (39).

In addition to the augmented hepatic synthesis and secretion of triglyceride, the removal of triglyceride from the plasma also has been suggested as a possible mechanism responsible for the hyperlipidemia associated with estrogen (12,13). A decrease in lipoprotein lipase activity and an enhanced adrenalin-induced lipolysis in the adipose tissue were reported in rats treated with estrogen (39,40). In humans, the activity of the hepatic triglyceride lipase in post-heparin plasma also has been shown to decrease following estrogen administration (41). However, decreases in those lipase activities do not seem to correlate well with triglyceride clearance from the plasma. In this study, although plasma triglyceride levels were markedly different between the control and E-treated chicks, no significant differences in triglyceride removal rates were observed. Similar findings have been reported in humans in which the clearance of exogeneously administered fat was unchanged following E treatment; when a combination of estrogen and progesterone was given, the clearance actually increased (9,10).

Now, the relationship between estrogen and coronary heart disease is highly controversial (42). However, since hyperlipidemia is a risk factor for coronary heart disease, the effect of estrogen-induced hyperlipidemia on the development of atherosclerosis deserves further attention.

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REFERENCES

1. Berkson, D.M., Stamler, J., and Cohen, D.B. (1964) *Clin. Obstet. Gynecol. 7,* 504-530.
2. Pick, R., Stamler, J., Rodbard, S., and Katz, L.N. (1952) *Circulation 6,* 858-861.
3. Kushwaha, R.S., and Hazzard, W.R. (1981) *Metabolism 30,* 359-366.
4. The Coronary Drug Project Research Group (1973) *J. Am. Med. Assoc. 226,* 652-657.
5. The Veterans Administration Cooperative Urological Research Group (1967) *Surg. Gynecol. Obstet. 124,* 1011-1017.
6. Stokes, T., and Wynn, V. (1971) *Lancet 2,* 677-680.
7. Reeves, R.D., Morris, M.D., and Barbour, G.L. (1976) *J. Am. Med. Assoc. 236,* 469-472.
8. Wynn, V., Mills, G.L., Doar, J.W.H., and Stokes, T. (1969) *Lancet 2,* 756-760.
9. Kekki, M., and Nikkila, E.A. (1971) *Metabolism 20,* 878-889.
10. Kissebah, A.H., Harrigan, P., and Wynn, V. (1973) *Horm. Metab. Res. 5,* 184-889.
11. Glueck, C.J., Fallet, R.W., and Scheel, D. (1975) *Metabolism 24,* 537-545.
12. Fabian, E., Stork, A., Kobilkova, J., and Sponarova, J. (1967) *Enzymol. Biol. Clin. 8,* 451-455.

13. Hazzard, W.R., Spiger, M.J., Bagdade, J.D., and Bierman, E.L. (1969) *N. Engl. J. Med. 280,* 471-474.
14. Kudzma, D.J., Hegstad, P.M., and Stoll, R.E. (1973) *Metabolism 22,* 423-434.
15. Kudzma, D.J., Swaney, J.B., and Ellis, E.N. (1979) *Biochim. Biophys. Acta 572,* 257-268.
16. Krauss, R.M., Lindgren, F.T., Wingerd, J., Bradley, D.D., and Sramcharan, S. (1979) *Lipids 14,* 113-118.
17. Smith, E.R., Damosa, D.A., and Davidson, J.M. (1977) *Methods Psycholbiol. 3,* 259-279.
18. Robertson, R.P., Gavareski, D.J., Henderson, J.D., Porte, D. Jr., and Bierman, E.L. (1973) *J. Clin. Invest. 52,* 1620-1626.
19. *Biological Handbooks: Respiration and Circulation* (1971) (Altman, P.L., and Dittmer, D.S., eds.) pp. 376-383, Federation of American Societies for Experimental Biology, Bethesda, MD.
10. Carlson, L.A., and Rossner, S. (1972) *Scan. J. Clin. Lab. Invest. 29,* 271-280.
21. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) *J. Clin. Invest. 34,* 1345-1353.
22. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
23. Noble, R.P. (1968) *J. Lipid Res. 9,* 693-700.
24. Allain, C.C., Poon, L., Chan, S.H., Richmond, W., and Fu, P. (1974) *Clin. Chem. 20,* 470-475.
25. Foster, L.B., and Dunn, R.T. (1973) *Clin. Chem. 19,* 338-340.
26. Folch, L., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
27. Eng, L.F., and Noble, E.P. (1968) *Lipids 3,* 157-162.
28. Cho, B.H.S., and Kummerow, F.A. (1978) *Biochem. Med. 20,* 267-278.
29. Dowdy, S., and Wearden, S. (1983) *Statistics for Research,* pp. 262-274, Wiley, New York.
30. Ferreri, L.F., and Naito, H.K. (1978) *Endocrinology 102,* 1621-1627.
31. Cho, B.H.S. (1981) Comp. *Biochem. Physiol. 68B,* 19-23.
32. Kudzma, D.J., St. Claire, F., DeLallo, L., and Friedberg, J. (1975) *J. Lipid Res. 16,* 123-133.
33. Aprahamian, S., Arslanian, M.J., and Stoops, J.K. (1979) *Lipids 14,* 1015-1020.
34. Mandour, T., Kissebah, A.H., and Wynn, V. (1977) *Eur. J. Clin. Invest. 7,* 181-187.
35. Chan., L., Bradley, W.A., Jackson, R.L., and Means, A.R. (1980) *Endocrinology 106,* 275-283.
36. Dashti, N., Kelley, J.L., Thayer, R.H., and Ontko, J.A. (1983) *J. Lipid Res. 24,* 368-380.
37. Kim, H.J., and Kalkhoff, R.K. (1978) *Metabolism 27,* 571-587.
38. Miller, N.E. (1978) *Lipids 13,* 914-919.
39. Kim, H.J., and Kalkhoff, R.K. (1975) *J. Clin. Invest. 56,* 888-896.
40. Tomita, T., Yonekura, I., Okada, T., and Hayashi, E. (1984) *Horm. Metab. Res. 16,* 525-528.
41. Applebaum, D.M., Goldberg, A.P., Pykalisto, D.J., Burnzell, J.D., and Hazzard, W.R. (1977) *J. Clin. Invest. 59,* 601-608.
42. Phillipis, G.B., Castelli, W.P., Abbott, R.D., and McNamara, P.M. (1983) *Am. J. Med. 74,* 863-869.

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Phospholipid Profile and Production of Prostanoids by Murine Colonic Epithelium: Effect of Dietary Fat[1]

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Dietary fat and abnormal production of various prostanoids have been linked to various disease states of the large bowel, including cancer of the colon. Studies were conducted to determine the effect of dietary fat (beef tallow or corn oil) on the lipid composition and prostanoid production of the murine colon.

Female C57BL/6J mice were fed high-fat (HF) diets (47% of calories as fat) or low-fat (LF) diets (10% of calories as fat). After four wk of dietary treatment, the mucosa was scraped, and lipids were extracted from the mucosal and muscle layers. The fat content of the diets did not significantly alter the amount of phospholipid (PL) or neutral lipid in the colonic tissue. However, the HF affected the PL profile of the colonic mucosa. For example, the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) was significantly higher for both the HF groups compared with that of the two LF groups (0.76 ± 0.15 and 0.80 ± 0.13 vs 0.31 ± 0.20 and 0.34 ± 0.18). Production of 13,14-dihydro-15-keto-PGE_2 (measured as bicyclic PGE_2) and TXB_2 (a stable metabolite of TXA_2) and $PGF_{1\alpha}$ (a stable metabolite of PGI_2) was unaffected by the dietary treatments. The muscle had a different PL profile (PC:PE is 2.6 ± 0.1) than the mucosa and contributed a larger proportion of the prostanoids formed. This study demonstrates that the phospholipid polar head group composition of normal colonic mucosa is altered by dietary fat, but the ability of the mucosa to synthesize metabolites of PGE_2, TXA_2 and PGI_2 is not affected.

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Epidemiological studies have suggested that there is a positive association between the consumption of dietary fat and mortality from colon cancer (1,2). The possible role of dietary fat in colon carcinogenesis also has received support from studies in animal models (3,4). However, not all dietary fats enhance colon tumorigenesis. High-fat diets containing coconut oil, olive oil or fish oil have no enhancing effect on colon tumor promotion (5,6). The varied effect of different types of fat on colon carcinogenesis suggests that the fatty acid composition of the diet may be an important factor in colon tumorigenesis.

The mechanism by which high-fat diets exert their effect on the colon is not understood. Many studies suggest that the effect of dietary fat on colon carcinogenesis is mediated through an increase in the concentration of fecal secondary bile acids (7,8). However, several investigators have failed to find such an association (9,10). It also has been hypothesized that high-fat diets may exert their effect by altering the membrane structure and function (11,12). Because fish oils inhibit colon tumorigenesis, prostanoids may be involved in mediating the fat effect (6,13). Various lines of evidence suggest that prostanoids may be implicated in tumor development (14-17). In spite of an association made between dietary fat, prostanoids and tumorigenesis in the colon, very little information is available regarding the influence of dietary fat on metabolism in the large bowel or in primary colonic tumors. Therefore, this study was designed to examine the effect of the type and level of dietary fat on lipid composition and prostanoid synthesis by the colon.

MATERIALS AND METHODS

Animals and diets. Female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) weighing 10-15 g were fed high-fat (HF) semipurified diets containing 47% of calories as fat or low-fat (LF) semipurified diets containing 10% of calories as fat. The fats used in this study were corn oil (CO) and beef tallow (BT). Sucrose was adjusted to equalize the caloric content of the diets. The fatty acid composition of the diets is shown in Table 1. The beef tallow was mixed with a small percentage of corn oil in order to meet the essential fatty acid needs of the animals. The mice were maintained in a temperature-controlled room (21 ± 1 C) with a 12 hr light-dark cycle. The mice were housed in plastic cages (5 mice per cage) with wire tops and sawdust bedding. Water and experimental diets were provided ad libitum. The diets were fed for four wk. After four wk of dietary treatment, the animals weighed 17.7 ± 0.4 g and 18.4 ± 0.6 g for low fat and high fat respectively.

Analysis of colonic lipids. The mice were killed by cervical dislocation, and 4 cm of colon from two mice were

TABLE 1

Fatty Acid Composition of the Diets

Major fatty acids % w/w	Percentage of total fatty acids			
	HFCO	HFBT	LFCO	LFBT
16:0	6.26	16.73	8.23	16.81
18:0	1.90	34.02	1.65	24.56
18:1	27.32	42.63	26.52	34.77
18:2	63.04	6.63	63.60	22.43

The high-fat diets contained 23.5% fat (w/w), and the low-fat diets contained 5% fat. Sufficient corn oil was added to the beef tallow diets to meet the essential fatty acid requirements of the animals. In the low-fat diets, sucrose was substituted for fat so that the caloric density of the essential nutrients remained the same.

[1]ARC Contribution No. 1504.

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Abbreviations: HF, high fat; HFBT, high-fat beef tallow; HFCO, high-fat corn oil; LF, low fat; LFBT, low-fat beef tallow; LTCO, low-fat corn oil; PC, phosphotidyl choline; PE, phosphotidyl ethanolamine; PGE_2, prostaglandine E_2; $PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; PGI_2, protacyclin; PL, phospholipid; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2

pooled for each sample prepared. The colons were removed and washed several times in Krebs Ringer to remove fecal material. The mucosa was scraped, and lipids were extracted from the mucosal and muscle layer by the method of Folch et al. (18).

The neutral lipids and the phospholipid classes were quantified by using thin layer chromatography performed on thin silica-coated quartz rods (Chromarod S., Technical Marketing Associates, Mississauga, Ontario, Canada) followed by flame ionization detection on an Iatroscan. The rods were saturated with copper sulphate (19).

For fatty acid analysis, phospholipids and triglycerides were separated by thin layer chromatography and fatty acid methyl esters were prepared with boron trifluoride methanol reagent (20). Fatty acid methyl esters were analyzed in a gas chromatograph (Hewlett Packard 5890A) on a fused silica capillary column, 30 m × 0.25 m). The gas chromatograph was equipped with a flame ionization detector and was programmed from 190–220 C at 10 C/min with a helium carrier gas flow rate of 45 cm/sec.

Measurement of prostanoids. After four wk of dietary treatment, the colon was removed, and the mucosal and muscle layers were incubated in a Krebs-Ringer solution for 30 min. The reaction was stopped with the addition of indomethacin (10 μg/ml). Stable metabolites of three prostanoids, prostaglandin E_2 (PGE_2), thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) were measured by radioimmunoassay using kits available commercially from Amersham (Amersham Corporation, Arlington Heights, IL). The assay systems combine the use of tritiated stable derivatives of prostanoids and antiserum specific for them. In the case of PGE_2, the assay system is based on the conversion of the major PGE_2 metabolite 13,14-dihydro-15-keto-prostaglandin E_2 to the stable, bicyclic compound 11-deoxy-13-14-dihydro-15-keto-11β, 16ξ-cyclo prostaglandin E_2. The cross reactivity of this radioimmunoassay with TXB_2 or PGF_1 is less than 0.0001%. TXA_2 and PGI_2 both are very unstable compounds and undergo spontaneous hydrolysis and form stable inactive metabolites thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$).

Statistical evaluation. The significance of dietary effects on the data was determined using Student's t-test.

RESULTS

Effect of diet treatment on colonic cell neutral lipid and phospholipid content. The dietary treatments did not result in a significant difference in the percentage of neutral lipid or in the percentage of cholesterol or cholesterol ester in the mucosa or muscle layers. The effect of dietary fat on the phospholipid content of the colonic mucosa and muscle is shown in Table 2. The level of fat in the diet had a significant effect on the relative amounts of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the colonic mucosa. When high-fat diets were fed, the ratio of PC:PE was 0.76 ± 0.15 and 0.80 ± 0.13 for the corn oil and beef tallow diets, respectively. Low-fat feeding resulted in a significantly lower PC:PE ratio for the LFCO and LFBT diets of 0.31 ± 0.20 and 0.34 ± 0.18, respectively.

The ratio of PC:PE was significantly higher in the muscle than in the mucosa, but there was no effect of the dietary treatments on the PC:PE ratio in the muscle (Table 3).

Effect of diet on phospholipid fatty acid composition of colonic mucosa and muscle. The fatty acid composition of the colonic mucosa phospholipids in the mice fed the different diets is shown in Table 4.

In the case of mucosal lipids, the type and level of dietary fat influenced the percent composition of only certain fatty acids. For example, the mucosal phospholipids of the beef tallow-fed animals (LFBT and HFBT) contained higher ($p \leq 0.05$) levels of oleic acid but not 18:0. In comparison, although the level of 18:2 was significantly higher in the HFCO group, generally the percent composition of 18:2 in mucosal phospholipids was less influenced by the level in the diet. Similarly, the level of 20:4 was unaffected by the dietary treatments (Table 4).

The colonic muscle phospholipids exhibited a slightly different pattern of fatty acid composition (Table 5). The level of 18:1 was higher in the beef tallow groups than in the high-fat corn oil group and was significantly lower than the level present in the mucosal phospholipids. Also, the levels of 18:2 and 20:4 were not related to the dietary treatments. Muscle phospholipids also contained substantially higher levels of 22:4 and 22:5 than those recorded for the mucosal fraction.

TABLE 2

Effect of Dietary Fat on the Phospholipid Content of the Colonic Mucosa

	% of total phospholipids			
	HFCO	HFBT	LFCO	HFBT
PC	35.3 ± 6.9	39.2 ± 9.9	20.2 ± 10.8	19.3 ± 6.7
PE	49.5 ± 12.3	50.2 ± 5.5	66.5 ± 10.0	64.6 ± 14.4
PC:PE	0.76 ± 0.15[b]	0.80 ± 0.13[b]	0.31 ± 0.20[a]	0.34 ± 0.18[a]
SM	5.3 ± 3.7	5.9 ± 6.5	3.3 ± 4.5	3.4 ± 5.9
Others	10.0 ± 5.2	4.6 ± 5.6	10.7 ± 3.2	12.6 ± 4.6

Values are means ± SEM; n = 5 for each group, and each n represents a pool of two mouse mucosas. Differences in horizontal means without a common superscript are statistically significant ($p \leq 0.05$). Rows without any superscripts contain values that are not significantly different.

PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin.

Effect of diet on triglyceride fatty acid content of colonic mucosa and muscle. The fatty acid composition of the colonic mucosa and muscle triglycerides is shown in Tables 6 and 7. The fatty acid profile was similar in the muscle and mucosal triglycerides. In both, the percentage of composition of triglyceride fatty acids only partially reflected the fat composition of the diets. For example, the beef tallow diets resulted in high levels of 18:1 but

TABLE 3

Effect of Dietary Fat on the Phospholipid Content of the Colonic Muscle

	% of total phospholipids			
	HFCO	HFBT	LFCO	LFBT
PC	47.0 ± 3.0	49.2 ± 6.1	47.9 ± 4.3	48.8 ± 2.8
PE	19.4 ± 3.5	18.7 ± 1.2	19.8 ± 3.9	18.1 ± 3.8
PC:PE	2.5 ± 0.4	2.7 ± 0.3	2.6 ± 0.8	2.8 ± 0.6
SM	13.3 ± 2.9	11.9 ± 2.5	15.8 ± 2.5	15.2 ± 4.6
Others	19.7 ± 3.1	19.3 ± 4.9	16.4 ± 3.9	17.7 ± 4.0

Values are means ± SEM; n = 5 for each group, and each n represents a pool of two mouse colonic muscles. Symbols of significance are the same as in Table 2.

TABLE 4

Effect of Diet on Fatty Acyl Components in Colonic Mucosa Total Phospholipids

% Fatty acid	HFCO	HFBT	LFCO	LFBT
14:0	0.88 ± 0.25	0.54 ± 0.04	0.98 ± 0.23	1.07 ± 0.08
16:0	18.89 ± 1.51^{b}	13.55 ± 1.23^{a}	16.40 ± 1.01^{b}	16.89 ± 0.34^{b}
16:1	1.79 ± 0.14	2.14 ± 0.31	2.64 ± 0.59	2.22 ± 0.28
18:0	16.78 ± 1.16	14.90 ± 0.13	14.59 ± 0.40	15.17 ± 0.44
18:1	14.77 ± 0.83^{a}	25.33 ± 0.93^{c}	18.78 ± 0.26^{b}	24.37 ± 0.67^{c}
18:2	12.82 ± 1.38^{c}	7.19 ± 0.35^{a}	8.15 ± 0.32^{b}	7.06 ± 0.31^{a}
18:3	0.33 ± 0.20^{a}	1.26 ± 0.37^{b}	0.59 ± 0.19^{a}	0.89 ± 0.26^{a}
20:3	11.45 ± 0.23^{b}	7.37 ± 0.91^{a}	8.31 ± 0.79^{a}	6.61 ± 0.35^{a}
20:4	21.05 ± 1.21	20.68 ± 1.57	22.43 ± 0.71	20.70 ± 1.03
22:4	2.45 ± 0.72	3.34 ± 0.65	3.58 ± 0.67	2.74 ± 0.77
22:5	2.70 ± 0.78	3.70 ± 1.40	3.54 ± 0.97	2.27 ± 1.05

Values given are means ± SEM; n = 5 for each group and each n represents a pool of two mouse colonic mucosas. Differences in horizontal means without a common superscript are statistically significant ($p \leq 0.05$). Rows without any superscripts contain values that are not significantly different. HFCO, high-fat corn oil; HFBT, high-fat beef tallow; LFCO, low-fat corn oil; LFBT, low-fat beef tallow.

TABLE 5

Effect of Diet on Fatty Acyl Components of Colonic Muscle Phospholipids

% Fatty acid	HFCO	HFBT	LFCO	LFBT
14:0	0.45 ± 0.12	0.64 ± 0.18	0.48 ± 0.08	0.63 ± 0.07
16:0	10.07 ± 0.50^{a}	11.92 ± 1.40^{a}	14.82 ± 0.49^{b}	17.81 ± 1.00^{c}
16:1	0.50 ± 0.10	1.05 ± 0.51	1.12 ± 0.27	1.17 ± 0.21
18:0	17.07 ± 0.51	18.23 ± 0.99	19.01 ± 0.66	19.69 ± 0.69
18:1	9.70 ± 0.31^{a}	17.47 ± 0.74^{c}	15.33 ± 0.62^{b}	19.00 ± 0.45^{c}
18:2	8.88 ± 0.50^{c}	5.28 ± 0.13^{a}	5.99 ± 0.28^{b}	6.13 ± 0.45^{b}
18:3	0.83 ± 0.02	1.23 ± 0.18	0.52 ± 0.21	0.47 ± 0.22
20:3	3.96 ± 0.41^{a}	3.86 ± 0.77^{a}	5.23 ± 0.47^{b}	4.14 ± 0.39^{a}
20:4	29.26 ± 0.32^{c}	25.51 ± 1.18^{b}	23.71 ± 0.52^{b}	21.96 ± 0.47^{a}
22:4	6.64 ± 0.48	4.98 ± 0.74	5.30 ± 0.58	3.59 ± 0.53
22:5	12.47 ± 0.82^{c}	9.82 ± 1.69^{b}	8.20 ± 0.77^{b}	5.42 ± 0.71^{a}

Values given are means ± SEM; n = 5 for each group, and each n represents a pool of two colonic muscles. Symbols of significance and abbreviations are the same as in Table 4.

TABLE 6

Effect of Diet on Fatty Acyl Components of Mucosal Triglycerides

% Fatty acid	HFCO	HFBT	LFCO	LFBT
14:0	0.16 ± 0.05	0.12 ± 0.02	0.13 ± 0.06	0.06 ± 0.02
16:0	9.88 ± 0.57[a]	14.89 ± 1.36[b]	14.95 ± 0.47[b]	14.86 ± 0.48[b]
16:1	0.19 ± 0.06[a]	0.90 ± 0.19[b]	1.80 ± 0.08[c]	2.02 ± 0.67[c]
18:0	3.63 ± 0.35[a]	6.60 ± 0.24[c]	3.74 ± 0.12[a]	4.95 ± 0.45[b]
18:1	34.90 ± 0.46[a]	68.56 ± 0.87[c]	53.76 ± 0.35[b]	66.48 ± 0.67[c]
18:2	46.63 ± 0.47[d]	8.43 ± 0.37[a]	26.45 ± 0.10[c]	11.45 ± 0.33[b]
18:3	0.58 ± 0.36	0.95 ± 0.58	ND	1.56 ± 0.64
20:3	0.52 ± 0.15	0.51 ± 0.43	0.43 ± 0.01	0.23 ± 0.05
20:4	1.57 ± 0.08	0.56 ± 0.09	1.10 ± 0.05	0.70 ± 0.04

Values given are means ± SEM; n = 5 for each group and each n represents a pool of two colonic mucosas. Symbols of significance are the same as in Table 4.

ND, not detectable; HFCO, high-fat corn oil; HFBT, high-fat beef tallow; LFCO, low-fat corn oil; LFBT, low-fat beef tallow.

TABLE 7

Effect of Diet on Fatty Acyl Components of Muscle Triglycerides

% Fatty acid	HFCO	HFBT	LFCO	LFBT
14:0	0.64 ± 0.20	0.67 ± 0.12	0.89 ± 0.37	0.54 ± 0.08
16:0	13.92 ± 2.78[a]	13.88 ± 0.52[a]	21.74 ± 3.38[b]	16.30 ± 2.87[a]
16:1	0.90 ± 0.20[a]	1.85 ± 0.16[b]	4.57 ± 0.78[c]	2.51 ± 0.69[b]
18:0	4.14 ± 0.21[a]	8.14 ± 0.36[c]	4.25 ± 0.11[a]	6.07 ± 0.42[b]
18:1	33.01 ± 1.11[a]	66.59 ± 0.81[c]	47.31 ± 2.09[b]	62.89 ± 1.79[c]
18:2	46.17 ± 1.56[c]	8.64 ± 0.43[a]	23.36 ± 1.10[b]	10.89 ± 0.94[a]
18:3	1.47 ± 0.22[b]	2.96 ± 0.23[c]	0.35 ± 0.15[a]	0.21 ± 0.06[a]
20:3	0.60 ± 0.15	0.23 ± 0.06	0.35 ± 0.15	0.21 ± 0.06
20:4	1.28 ± 0.33	0.56 ± 0.15	1.21 ± 0.13	0.63 ± 0.16

Values given are means ± SEM; n = 5 for each group and each n represents a pool of two colonic muscles. Symbols of significance are the same as in Table 4.

HFCO, high-fat corn oil; HFBT, high-fat beef tallow; LFCO, low-fat corn oil; LFBT, low-fat beef tallow.

TABLE 8

Effect of Dietary Fat on Prostanoid Production by Colonic Mucosa and Muscle

	HFCO	HFBT	LFCO	LFBT
Mucosa				
Bicyclic PGE_2	3.15 ± 0.44	2.2 ± 0.52	2.0 ± 0.34	3.73 ± 1.16
TXB_2	0.66 ± 0.05	0.66 ± 0.07	0.51 ± 0.12	1.11 ± 0.34
$PGF_{1\alpha}$	2.44 ± 0.25	2.31 ± 0.58	2.59 ± 0.59	3.78 ± 0.90
$PGF_{1\alpha}$:TXB_2	3.84 ± 0.62	3.31 ± 0.72	3.83 ± 0.70	4.24 ± 1.40
Muscle				
Bicyclic PGE_2	3.82 ± 0.40	3.64 ± 0.24	3.52 ± 0.21	5.06 ± 0.78
TXB_2	1.43 ± 0.10	1.55 ± 0.16	1.42 ± 0.24	1.90 ± 0.28
$PGF_{1\alpha}$	16.68 ± 1.07	18.83 ± 2.04	13.21 ± 1.60	16.57 ± 3.59
$PGF_{1\alpha}$:TXB_2	11.70 ± 0.37	12.22 ± 0.78	9.70 ± 1.02	8.87 ± 1.30

Values given are means ± SEM; n = 5 for each group and each n represents either one mucosa or one muscle. Values expressed as ng × 10^3/g/30 min. Symbols of significance are the same as in Table 4.

PGE_2, prostaglandin E_2; TXB_2, thromboxane B_2; $PGF_{1\alpha}$, prostaglandin $F_{1\alpha}$; HFCO, high-fat corn oil; HFBT, high-fat beef tallow; LFCO, low-fat corn oil; LFBT, low-fat beef tallow.

only small increases in 18:0 in the triglycerides. Corn oil feeding resulted in higher levels of 18:2 in colonic triglyceride.

Effect of dietary fat on prostanoid production by murine colon. The effect of dietary fat on the production of TXB_2, $PGF_{1\alpha}$ and bicyclic PGE_2 is shown in Table 8. The dietary treatments did not result in a significant difference in the production of TXB_2, $PGF_{1\alpha}$ or PGE_2 by either the mucosa or the muscle. However, in each case the muscle layer contributed a larger percentage of the total prostanoids formed.

DISCUSSION

In this study, we examined the effect of two different types of fat (corn oil and beef tallow) that vary widely in their fatty acid composition.

Beef tallow is high in saturated fatty acids (16:0, 18:0), while corn oil is high in the polyunsaturated fatty acid 18:2. However, both types of fat promote colon tumorigenesis when they are included in the diet at high levels (5). In view of the similar effects of these fats on colon carcinogenesis, we wanted to determine whether high-fat feeding of these two fats would result in common compositional and biochemical alterations. The results of this study indicate that the type of fat fed does not affect the phospholipid content of the colonic mucosa or muscle. However, the quantity of fat in the diet appears to be a significant determinant of the phospholipid profile of the colonic musosa. Mice fed the high-fat diets had significantly higher ratios of mucosal PC:PE. It is possible that a change in the polar head group composition may result in a change in the function of the membrane in vivo (21). The small polar head group of phosphatidylethanolamine allows very close packing and reduces the permeability and fluidity of the membrane (22). Any changes in the fluidity of the membrane caused by increased PC could to some extent depend on the fatty acid composition of the newly synthesized PC. However, it is not possible to conclude from this study if the plasma membrane composition of mucosal cells was altered or if the phospholipid changes reflect differences in the amount of certain organelles in the colonocytes. Another limitation of this study is that although the fecal material was removed and the colons were rinsed very thoroughly, it still is possible that bacteria may remain adhered to the mucosa, thus contaminating the lipid sample.

In mammals, changes in membrane lipid composition have been achieved by feeding different dietary fats (23,24). The activity of lipid-dependent enzymes may be dependent on the polar head group composition of membranes (25) or on the fatty acid composition of the membrane (26). Changes in membrane fluidity have been shown to influence the conformation of the active site of some membrane-associated enzymes (27).

The fluidity and permeability of biological membranes also is determined by the fatty acid composition of phospholipids of cell membranes (28). The phospholipid fatty acid composition reflected only partially the composition of dietary fat. Overall, the levels of 18:0 and 20:4 were unaffected by dietary treatments. This suggests and supports the findings of others that a homeostatic mechanism is operative in biological membranes with respect to fatty acid composition (29). This mechanism may be effective in maintaining a certain level of unsaturation in the membrane lipids. It previously has been observed in platelets and other tissues that an increase of 18:2 over a threshold level may not lead to a further increase in membrane arachidonic acid (30).

Dietary fat did not change the level of the prostanoids that were measured in this study. Bicyclic PGE_2, TXB_2 and $PGF_{1\alpha}$ were measured because colonic carcinomas have been found to synthesize increased levels of PGE_2 (15). In addition, TXA_2 or its active metabolite stimulate tumor cell replication, whereas prostacyclin or its active metabolite inhibits tumor cell growth (17). We hypothesized that a high-fat diet would alter the synthesis of these prostanoids in the colonic tissue. However, dietary fat irrespective of the type and level did not influence synthesis of any of these compounds by the colonic mucosa or the muscle layer. This is interesting in view of a high-fat diet increasing the relative proportion of PC in the mucosal cells but unable to change the level of arachidonic acid in the membrane lipids of colonic mucosa and muscle layers. Because the level of arachidonic acid in the membrane was not different between the diets, it might be expected that no difference would occur in the production of prostanoid metabolites at this site. Another noteworthy finding is that in comparison to the mucosa, the muscle layer did contain higher levels of arachidonic acid in the phospholipids. Furthermore, muscle synthesized significantly higher levels of prostanoids studied, especially TXB_2 and $PGF_{1\alpha}$. This observation corroborates the findings that colonic muscle synthesizes significantly more prostanoids than the mucosal layer (31). These observations suggest that prostanoid synthesis is dependent on the local requirement of the organ. These observations are made on total colonic mucosa or muscle, and no distinction was made between the cell types. It may be of interest in future studies to compare the proliferative compartment with the nonproliferative compartment.

The mechanism by which prostanoid production is regulated in normal or pathological situations in the colon is not known. However, it is apparent that many disease states including cancer, do lead to altered production of certain prostanoids in the gastrointestinal tract (32). Whether the level and type of fat will affect prostanoid production in the neoplastic colon and whether certain prostanoids are causally related to cancer remains to be investigated. Further studies are in progress to investigate the effect of dietary fats on the normal and neoplastic colons.

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REFERENCES

1. Wynder, E.L., and Reddy, B.S. (1974) *Cancer 34,* 801-806.
2. Carroll, K.K., and Khor, H.T. (1975) *Prog. Biochem. Pharmacol. 10,* 308-353.
3. Reddy, B.S., Watanabe, K., and Weisburger, J.H. (1977) *Cancer Res. 37,* 4156-4159.
4. Bull, A.W., Soullier, B.K., Wilson, P.S., Hayden, M.T., and Nigro, N.D. (1979) *Cancer Res. 39,* 4956-4959.
5. Reddy, B.S., and Maeura, Y. (1984) *J. Natl. Cancer Inst. 72,* 745-750.

6. Reddy, B.S., and Maruyama, H. (1986) *Cancer Res. 46,* 3367-3370.
7. Narisawa, T., Reddy, B.S., and Weisburger, J.H. (1978) *Gastroenterol. Jap. 13,* 206-212.
8. Weisburger, J.H., Reddy, B.S., Barnes, W.S., and Wynder, E.L. (1983) *Env. Health Persp. 50,* 101-107.
9. Hikasa, Y., Tanida, N., Ohno, T., and Shimoyama, T. (1984) *Gut 25,* 833-838.
10. Mudd, D.G., McKelvey, S.T.D., Norwood, W., Elmore, D.T., and Roy, A.D. (1980) *Gut 21,* 587-590.
11. Hopkins, G.J., and West. C.E. (1976) *Life Sci. 19,* 1103-1116.
12. Fischer, P.B., Cogan, U., Horowitz, A.D., Schacter, D., and Weinstein, I.B. (1981) *Biochem. Biophys. Res. Commun. 100,* 370-376.
13. Carroll, K.K. (1986) *Lipids 21,* 731-732.
14. Form, D.M., Sidky, Y.A., Kubai, L., and Auerbach, R. (1982) in *Prostaglandins and Cancer: First International Conference,* pp. 685-689, Alan R. Liss Inc., New York.
15. Jaffe, B.M. (1974) *Prostaglandins 6,* 453-461.
16. Bennett, A. (1982) in *Prostaglandins and Cancer: First International Conference,* pp. 759-766, Alan R. Liss Inc., New York.
17. Honn, K.V., and Meyer, J. (1981) *Biochem. Biophys. Res. Comm. 102,* 1122-1129.
18. Folch, J., Lees, M., and Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
19. Kramer, J.K.G., Fouchard, R.C., and Farnworth, E.R. (1986) *J. Chromatogr. 351,* 571-573.
20. Morrison, W.R., and Smith, C.M. (1964) *J. Lipid Res. 5,* 600-608.
21. Fourcans, B., and Jain, M.K. (1974) *Adv. Lipid Res. 12,* 148-226.
22. Phillips, M.C., Finer, E.G., and Hauser, H. (1972) *Biochim. Biophys. Acta 290,* 397-402.
23. Innis, S.M., and Clandinin, M.T. (1981) *J. Biochem. 198,* 231-234.
24. Robblee, N.M., and Clandinin, M.T. (1984) *J. Nutr. 114,* 263-269.
25. Sandermann, H. (1978) *Biochim. Biophys. Acta 515,* 209-237.
26. Bloj, B., Morero, R.D., Farias, R.N., and Trucco, R.E. (1973) *Biochem. Biophys. Acta 311,* 67-69.
27. McMurchie, E.J., and Raisin, J.K. (1979) *Biochim. Biophys. Acta 554,* 364-374.
28. Van Golde, L.M.G., and Van Deenen, L.L.M. (1966) *Biochim. Biophys. Acta 125,* 496-509.
29. Stubbs, C.D., and Smith, A.D. (1984) *Biochim. Biophys. Acta 779,* 89-137.
30. Galli, C. (1980) in *Advances in Nutritional Research* (Draper, H.H., ed.), Vol. 3, pp. 95-126, Plenum Press, New York.
31. Craven, P.A., and DeRubertis, F.R. (1983) *Prostaglandins 26,* 583-604.
32. Bennett, A. (1976) in *Advances in Prostaglandin and Thromboxane Research* (Samuelson, B., and Paoletti, R., eds.), Vol. 2, pp. 547-555, Raven Press, New York.

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Characterization of HETEs and Related Conjugated Dienes by UV Spectroscopy

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Three distinct pairs of HETEs can be distinguished on the basis of their UV spectra. We used hydroxy-linoleates (hydroxy-octadeca-*cis-trans*-dienoates) as a base for comparisons; both the 9- and 13-hydroxy isomers have identical chromophores with λ_{max} near 234 nm. The presence of a double bond three carbons removed from the conjugated diene (the chromophore of 9- and 11-HETE) causes a shift in the observed λ_{max} to near 235 nm. A double bond β to the chromophore (5- and 15-HETE) gives a further shift of 1.5 nm, giving a λ_{max} between 236–236.5 nm. With double bonds in both these positions (8- and 12-HETE), the λ_{max} is observed near 237 nm. It is apparent that the exact λ_{max} of the *cis-trans* diene chromophore is influenced in a consistent way by the adjacent methylene interrupted *cis* double bonds.
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The λ_{max} of the UV chromophores in HETEs and related hydroxy dienes usually is quoted in the range of 234–237 nm. It is well recognized that these *cis-trans* conjugated hydroxy dienes can be distinguished from the *trans-trans* analogues (λ_{max} 230–232 nm).

We noticed that there are more subtle but reproducible differences in the chromophore of the individual HETEs. Here we report that the precise λ_{max} and shape of the HETE chromophore depends on the arrangement of the adjacent *cis* double bonds and that three pairs of HETEs can be distinguished by their UV spectra.

METHODS

Preparation and purification of HETEs. HETEs were prepared by autoxidation of 100 mg quantities of arachidonic acid with α-tocopherol (1). Briefly, the arachidonic acid was mixed with 10% w/w α-tocopherol (Sigma Chemical Co., St. Louis, MO) and left under an oxygen atmosphere for three days at 37 C. After three days, the autoxidation mixture contained 15–20% hydroperoxides. Before HPLC purification, the hydroperoxides were reduced with a molar excess of triphenylphosphine in methanol. The HETEs then were separated on an Alltech Semi-Prep silica column (250 × 10 mm) and further purified on an Alltech Analytical silica column (250 × 4.6 mm) using a solvent of hexane/isopropanol/glacial acetic acid (100/1.6/0.1, v/v/v). Additional RP-HPLC separation and purification of the 12- and 15-HETEs was performed on an Alltech Semi-Prep C-18 column. Concentrated solutions of pure HETEs (0.2–5 mg/ml) were stored in methanol or ethanol under argon at −70 C.

The 9-hydroxy and 13-hydroxy-octadecadienoates were prepared by the same method. The pair with the *cis-trans* conjugated diene was obtained by autoxidation of the methyl ester of linoleic acid, and the *trans-trans* pair from methyl linoelaidate (9*trans*,12*trans*-18:2).

HHT was recovered from an incubation of arachidonic acid with human platelets and was purified by RP- and SP-HPLC. All the standards were identified by gas chromatography-mass spectrometry (2,3).

Recording of UV spectra. A Beckman DU-7 scanning spectrophotometer was used with the standard slit. The resolution is quoted by the manufacturers as 2 nm, and can be judged from the appearance of the two small UV peaks in the Holmium oxide filter, Figure 1.

Concentrated solutions of pure conjugated dienes (0.2–5 mg/ml) were diluted in ethanol, and the concentration was carefully adjusted to give an absorbance of exactly 0.90 AU (ca. 12 μg/ml, assuming ε = 23,000 [4]). The solutions were scanned from 300–200 nm at 300 nm/min with ethanol as background. The spectra were stored in memory and plotted together as shown in the figures. The calculation of the first derivative of absorbance included a smoothing factor (delta λ); this was set at 0.8 nm, the lowest possible value compatible with the scanning speed. "Delta λ" is the range of wavelength used in the point by point measurement of slope for calculation of $dA/d\lambda$.

RESULTS AND DISCUSSION

UV spectra of hydroxy-18:2. The 9- and 13-hydroxy derivatives of linoleic acid represent the basic *cis-trans* conjugated diene (with no adjacent nonconjugated double

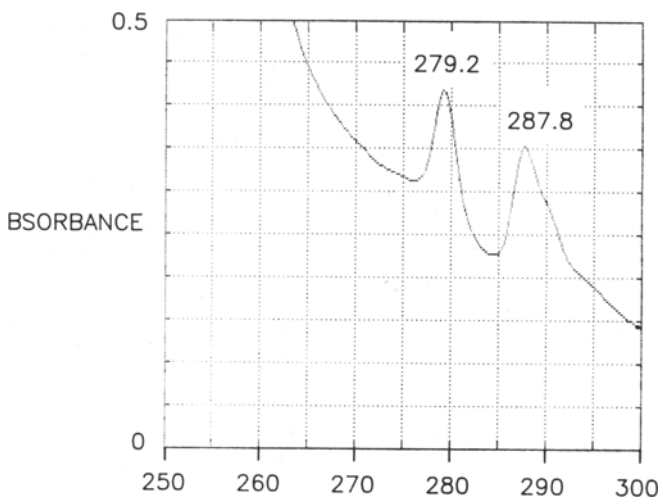

FIG. 1. UV spectrum of Holmium oxide filter on the Beckman DU-7.

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Abbreviations: HETE, hydroxyeicosatetraenoic acid; HHT, 12-hydroxy-heptadeca-5,8,10-(*cis,trans,trans*)-trienoic acid; hydroxy-linoleate, describes the two primary products of linoleate oxygenation, more properly named 9-hydroxy-octadeca-10*trans*,12*cis*-dienoate and 13-hydroxy-octadeca-9*cis*,11*trans*-dienoate. Hydroxy-linoelaidate refers to the corresponding *all-trans* dienes. RP-HPLC, reversed-phase high pressure liquid chromatography; SP-HPLC, straight-phase high pressure liquid chromatography.

bonds). They have identical UV spectra with λ_{max} near 234 nm. The corresponding *trans-trans* analogues are another identical pair with a λ_{max} 3 nm lower, near 231 nm. Figure 2 depicts the four spectra over a 50 nm range (215–265 nm).

The exact λ_{max} can be determined by inspection of the first derivative of the chromophore. The point at which the first derivative crosses zero corresponds to $dA/d\lambda = 0$, and consequently equals the λ_{max}. Figure 3a shows the first derivative of the entire chromophore of the four hydroxy-octadecadienoates (215–265 nm). Figure 3b focuses on the range 230–240 nm, allowing the precise λ_{max} to be measured.

UV spectra of HETEs. An overlay of the spectra of six HETEs (5-, 8-, 9-, 11-, 12-, and 15-HETE) is shown in Figure 4. It is apparent that there are only three different chromophores. Each represents a pair of HETEs: 9- and 11-HETE have the lowest λ_{max} (near 235 nm), the middle pair is 5- and 15-HETE (λ_{max} 1.5 nm higher), and 8- and 12-HETE have the highest λ_{max}, near 237 nm. Again, the exact λ_{max} can be determined by looking at the first derivative of the chromophore (Fig. 4b). This also emphasizes the three distinct pairs of HETEs.

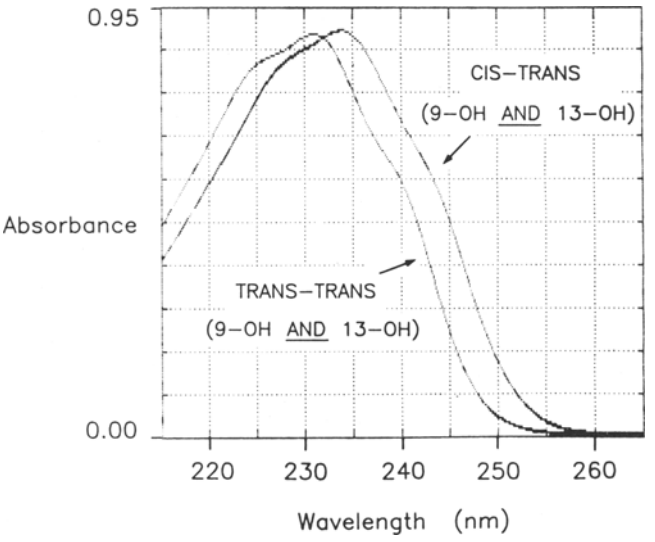


FIG. 2. UV spectra of four conjugated dienes: 9- and 13-hydroxy linoelaidates *(trans-trans)* and 9- and 13-hydroxy linoleates *(cis-trans).*

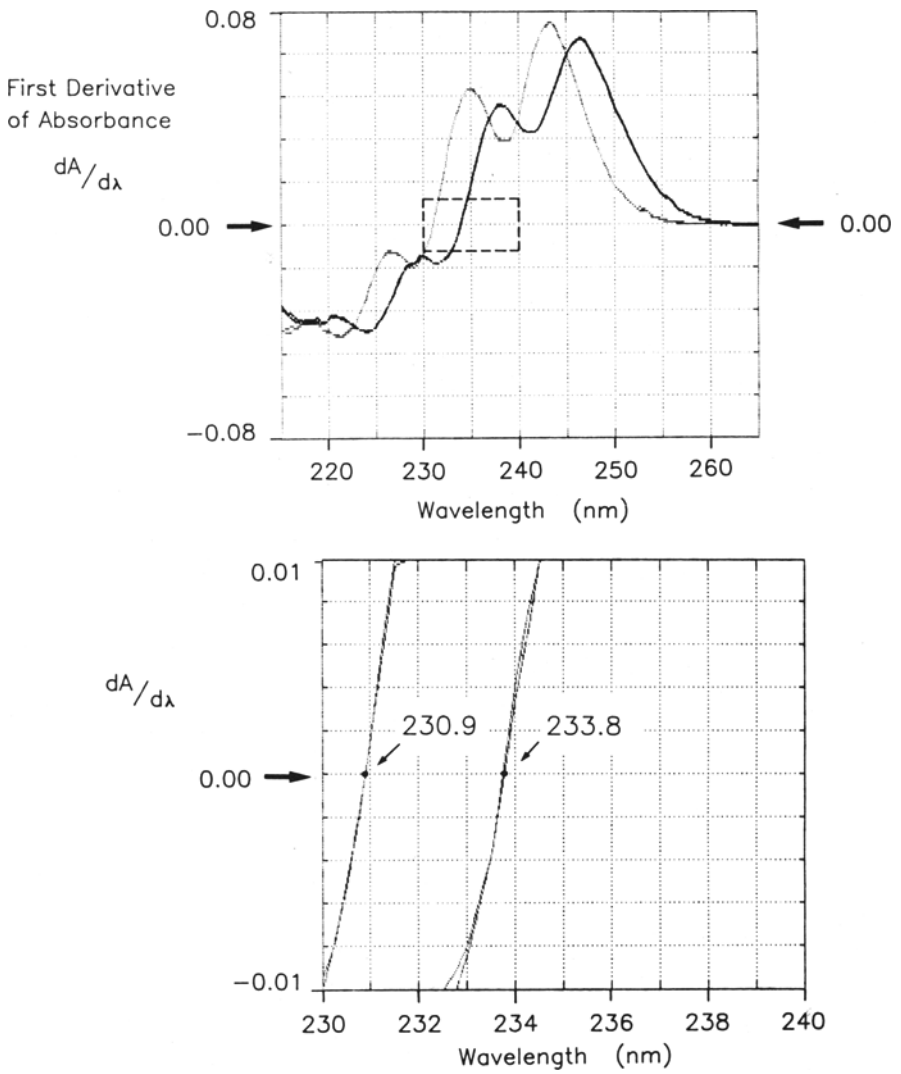


FIG. 3. First derivative of absorbance of compounds shown in Figure 2. Top, 215–265 nm; below, expansion of the boxed area, 230–240 nm.

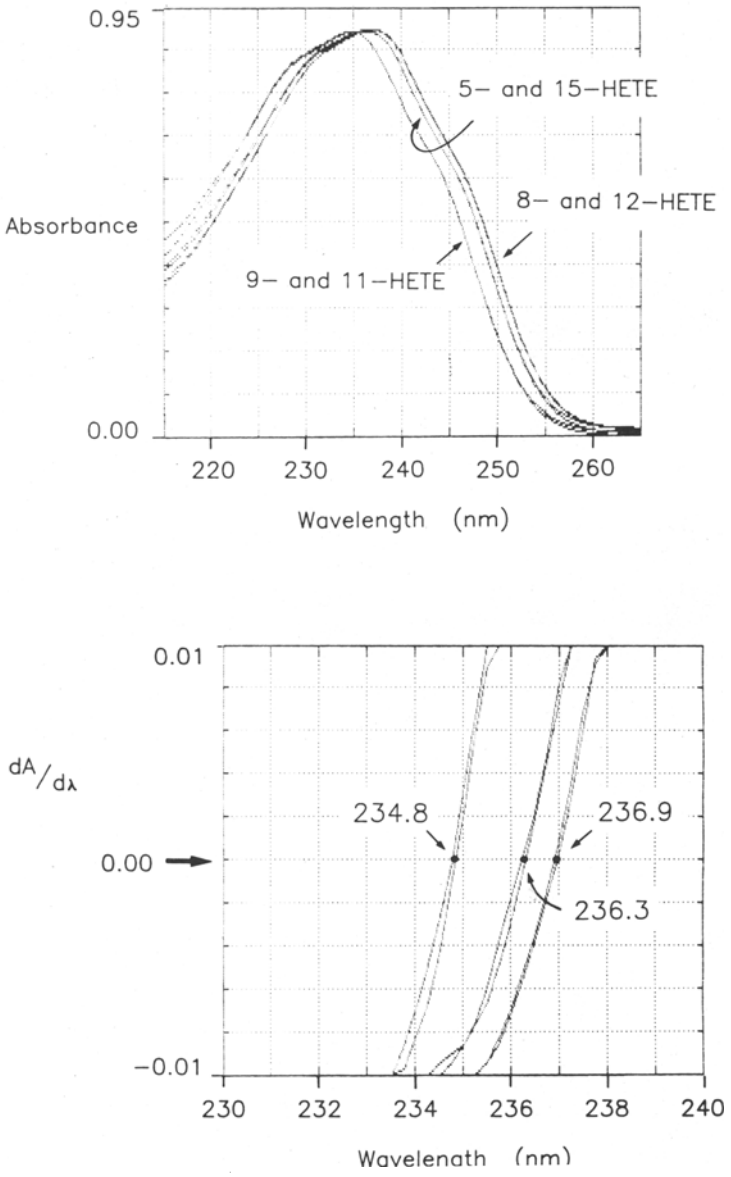


FIG. 4. Overlay of UV spectra of six HETEs. Top, absorbance spectra; below, expanded view of first derivative.

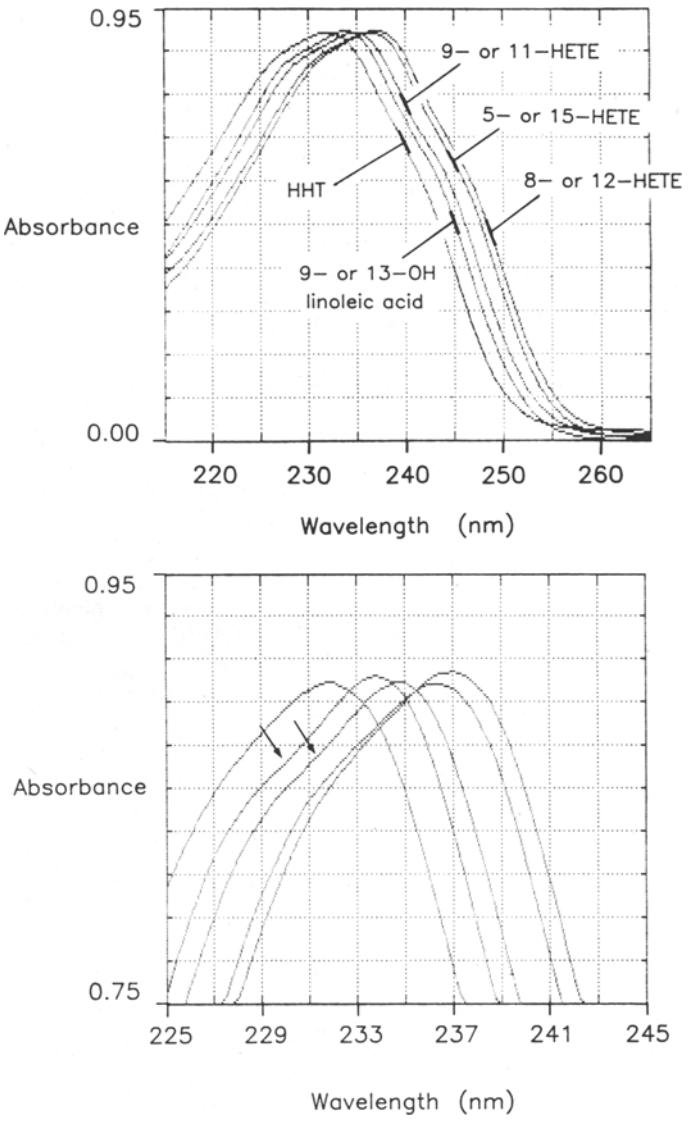


FIG. 5. Comparative shapes of UV spectra of conjugated dienes. Top, absorbance spectra 215–265 nm; below, expansion near the λ_{max}; arrows highlight the more distinct shoulder in the hydroxy-octadecadienoates and in 9- or 11-HETE. The *trans-trans* hydroxy-octadecadienoates (not shown in this figure) also have a distinct hypsochromic shoulder.

Shape of chromophore. At wavelengths above the λ_{max}, the conjugated diene chromophores are identical in shape. This is evident as the "parallel" nature of the chromophores in Figure 5. There are subtle differences in the shoulder just below the λ_{max}; there is a more distinct "notch" in the spectra of the hydroxy-octadecadienoates and in 9- and 11-HETEs (arrows in Fig. 5b). This figure also emphasizes the assymetrical character of the top of the conjugated diene chromophore.

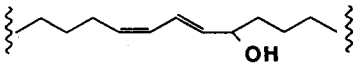
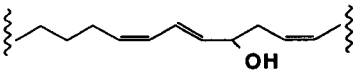
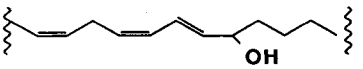
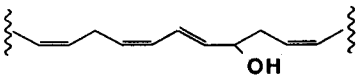
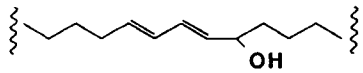
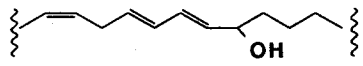
"Rules" of diene absorbance. The structures of several conjugated dienes and their λ_{max} are listed in Table 1. The adjacent "non-conjugated" double bonds in the chromophore shift the λ_{max} from the base value of hydroxy-linoleate (233.8 nm): a double bond three carbons from the conjugated diene gives an increase of 1.0 nm (9- and 11-HETE); a double bond two carbons removed from the diene increases the base value 2.5 nm (5- and 15-HETE); a diene with double bonds on both sides of the conjugated diene increases the base value 3.1 nm (8- or 12-HETE).

Lack of additive effects. If the effects of double bonds on both sides of the chromophore were additive, the predicted value for 8- and 12-HETEs would be 3.5 nm above base (1 + 2.5 nm). The observed increase (3.1 nm) falls slightly short of this value.

Differences for cis-trans *and* trans-trans *systems.* The difference in the λ_{max} of the *cis-trans* and *trans-trans* hydroxy-octadecadienoates is 3 nm. The only other *trans-trans* conjugated diene we compared was HHT. Based on the observed λ_{max} of HHT, it would appear that the "rules" that govern the position of λ_{max} are different for *trans-trans* and *cis-trans* conjugated dienes. HHT has a

TABLE 1

Influence of "Nonconjugated" Double Bonds on the Diene Chromophore

Chromophore	Compound	λ_{max}	λ_{max} Relative to base value
	9- or 13-hydroxy linoleate	233.8	± 0 = base
	9- or 11-HETE	234.8	+ 1.0
	5- or 15-HETE	236.3	+ 2.5
	8- or 12-HETE	236.9	+ 3.1
	9- or 13-hydroxy-linoelaidate	230.9	± 0 = base *(trans/trans)*
	HHT	231.8	+ 0.9 nm *(trans/trans)*

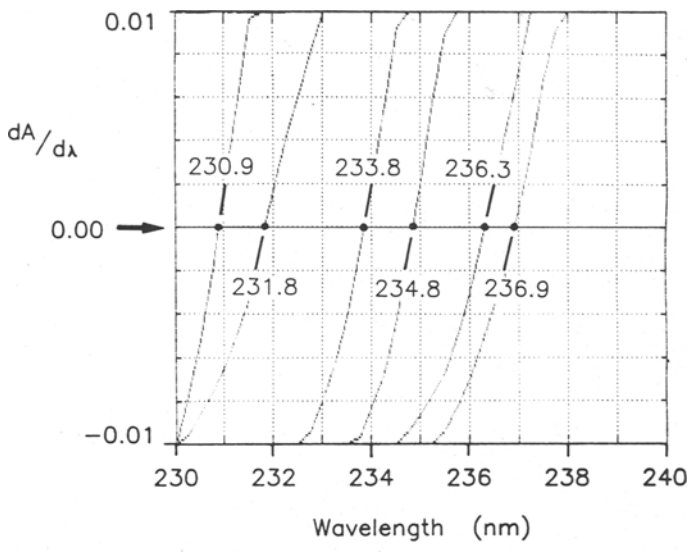


FIG. 6. Comparison of the λ_{max} of different conjugated dienes. In order of increasing λ_{max}, the compounds are hydroxy-linoelaidate (230.9 nm), HHT (231.8 nm), hydroxy-linoleate (233.8 nm), 9- or 11-HETE (234.8 nm), 5- or 15-HETE (236.3 nm), and 8- or 12-HETE (236.9 nm).

double bond two carbons from the conjugated *trans-trans* diene. Using the observations on the *cis-trans* dienes, the expected increase above base value due to the double bond is 2.5 nm (c.f. 5- and 15-HETE). The base value of a *trans-trans* conjugated diene is 230.9 nm, giving a predicted λ_{max} of HHT of 233.4 nm (230.9 + 2.5 nm). This is considerably different from the observed value for HHT (231.8 nm). The relative separations are summarized in Figure 6.

Application to other polyunsaturated fatty acids. We have not completely characterized the UV spectra of related hydroxy dienes, but we have seen evidence that the general trends in these rules do apply to other compounds. For example, in the series of hydroxy-eicosapentaenoic acid derivatives, the 9- and 14-hydroxy-eicosapentaenoates have the same arrangement of the double bonds around the diene as do 9- and 11-HETEs. These two EPA derivatives have a λ_{max} near 235 nm. In terms of the UV chromophore, the 11-hydroxy-eicosapentaenoate is an analog of 8- or 12-HETE, and its λ_{max} is near 237 nm (5). These observations fit with the "rules" in Table 1.

Caveats in identification of HETEs: instrument calibration and precision. The precise value of λ_{max} varies with calibration of the spectrophotometer, and a set of appropriate HETE standards are needed for direct comparisons. We want to emphasize that the precise values we quote here may vary from values obtained on another instrument. We used the phrase "near 235 nm" to allow for this. Over the course of months, the values found on our own instrument may vary by 0.5 nm, although the differences between individual spectra remain unchanged. Changes in resolution (slit width) of the spectrophotometer also can cause very slight changes in the observed λ_{max}. This relates to the assymetric character of the HETE chromophore (Fig. 5b): as the slit is widened, the higher absorbance on the hypsochromic side results in a bias which shifts the observed λ_{max} to lower wavelength. In the Beckman DU-7 spectrophotometer, there is a closely related artifact in the calculation of the first derivative of absorbance. The software uses a smoothing factor called "delta λ" that averages the calculation of dA/dλ over a chosen range of wavelength (0.2–16 nm). The use of even relatively low values of "delta λ" (0.8–2 nm) smooths the curve but also causes

a slight shift of the apparent λ_{max} ($dA/d\lambda = 0$) to lower wavelength.

Caveats in identification of HETEs: requirement for pure compounds. Because the diene chromophore is near the lower end of the UV, slight differences in end absorption can change the apparent λ_{max}. High end absorption will shift the apparent λ_{max} to lower wavelength, while oversubtraction of background can have the opposite effect. It is difficult to obtain a quality spectrum on less than 5–10 μg of pure compound. With smaller amounts of material, the most reliable spectra can be obtained with an on-line detector on HPLC.

ACKNOWLEDGMENTS

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REFERENCES

1. Peers, K.E., and Coxon, D.T. (1983) *Chem. Phys. Lipids 32,* 49–56.
2. Boeynaems, J.M., Brash, A.R., Oates, J.A., and Hubbard, W.C. (1980) *Anal. Biochem. 104,* 259–267.
3. Hubbard, W.C., Hough, A.J., Brash, A.R., Watson, J.T., and Oates, J.A. (1980) *Prostaglandins 20,* 431–437.
4. Gibian, M.J., and Vandenberg, P. (1987) *Anal. Biochem. 163,* 343–349.
5. Hawkins, D.J., and Brash, A.R. (1987) *J. Biol. Chem. 262,* 7629–7634.

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Inhibition of Cytochrome c Oxidase and Hemolysis Caused by Lysosphingolipids

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Galactosylsphingosine, glucosylsphingosine and sphingosine all inhibited cytochrome c oxidase activity in mitochondria from rat liver; more than 50% inhibition was caused by 5 μM lipid (0.1 μmol/mg mitochondrial protein). However, these lysosphingolipids did not suppress the activity of purified cytochrome c oxidase. When the enzyme was "reconstituted" with phosphatidylcholine, the lysosphingolipids clearly inhibited the activity. On the other hand, galactosylsphingosine, glucosylsphingosine and sphingosine all hemolyzed erythrocytes, indicating that lysosphingolipids can disrupt the membrane. Thus, it appears that the inhibition of cytochrome c oxidase, a membrane-bound enzyme in mitochondria, is due to perturbation of the environment of the enzyme and that the primary attacking site of the lysosphingolipids is the membrane. Because the potency to inhibit cytochrome c oxidase and to hemolyze erythrocytes did not differ among these lysosphingolipids and because galactosylceramide caused neither inhibition of cytochrome c oxidase nor hemolysis, the free amino group in the lysosphingolipids seems to be essential to give the effects. In addition, both inhibition of cytochrome c oxidase and hemolysis caused by lysosphingolipids were completely abolished by albumin, suggesting that toxic effects of lysosphingolipids may not be apparent in blood.
Lipids 23, 345-348 (1988).

Although enzymatic defects have been well-established in most sphingolipidoses, including Krabbe and Gaucher disease (1,2), it has not been defined how the defect of an enzyme causes the pathology. In Krabbe disease (globoid cell leukodystrophy), a striking accumulation of galactosylsphingosine (psychosine), which is a natural substrate of the missing enzyme (3,4), has been demonstrated (5,6). Since galactosylsphingosine is cytotoxic, the devastating pathology in Krabbe disease may be explained by the toxic effects of the accumulated lipid (6). In Gaucher disease, accumulation of another lysosphingolipid (glucosylsphingosine) has been shown (7) and a similar pathophysiology as in Krabbe disease has been suspected. However, the mechanism of cytotoxicity of these lysosphingolipids is not known.

We previously have noted that galactosylsphingosine is a powerful inhibitor of cytochrome c oxidase in mitochondria (8). In this study, we have examined whether glucosylsphingosine and sphingosine have similar effects of galactosylsphingosine. Furthermore, we studied the effects of these lysosphingolipids on cytochrome c oxidase, either in purified or in "reconstituted" form and their effects on erythrocytes compared with those of galactosylceramide.

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Abbreviation: HPTLC, high performance thin layer chromatograph.

MATERIALS AND METHODS

Materials. Galactosylsphingosine, glucosylsphingosine, sphingosine, galactosylceramide (cerebroside from bovine brain, "lower spot") and γ-globulin (from human serum) were purchased from Sigma Chemical Co. (St. Louis, MO), crystalline human serum albumin from Chemical Dynamics Corporation (South Plainfield, NJ), phosphatidylcholine (from egg) from Koch-Light (Cohnbrook Berks, England) and cytochrome c from Boehringer-Mannheim-Yamanouchi (Tokyo, Japan). Ion exchange cellulose (DE-52) was from Whatman KK (Tokyo, Japan) and Sephacryl S-300 gel from Pharmacia KK (Tokyo, Japan). All other chemicals were reagent grade. The reduced form of cytochrome c was prepared as described (8).

Centrifree (centrifuging ultrafiltration apparatus) was purchased from Amicon (Danvers, MA), Sep-Pak cartridge from Waters (Milford, MA), and HPTLC (high performance thin layer chromatograph) plate (silica gel 60) from E. Merck (Darmstadt, West Germany). Borate impregnation of the HPTLC plate was done as described (9).

Preparation of samples. Mitochondria from the liver of rats (Wistar, male) were prepared by a slightly modified method (8) of Borgese and Meldolesi (10). The mitochondria were kept on ice (for cytochrome c oxidase measurement) or at −80 C (for cytochrome c oxidase purification).

Purification of cytochrome c oxidase was done following the method of Merle and Kadenbach (11). After final purification by gel filtration, the peak fraction was collected and frozen in liquid nitrogen without further concentration of the enzyme. The enzyme was kept at −80 C until use.

The purified enzyme was activated by phosphatidylcholine as follows. Phosphatidylcholine (0.1 mg) in chloroform/methanol (2:1, v/v) was dried as a thin film under a stream of nitrogen. After 1 ml of the buffer (50 mM glycylglycine buffer [pH 7.4] containing 3.1 mM Tris-HCl [pH 7.4] and 2 mM EDTA-2K) was added and vortexed vigorously for 30 seconds, the solution was sonicated in a water-bath type sonicator for two min. The solution (0.0426 ml) was cooled in ice, and 0.025 ml (6.75 μg protein) of the purified enzyme was added and vortexed for 30 seconds. The mixture was left on ice for one hr and diluted with 0.1825 ml of the buffer.

Human blood was obtained from a healthy male and heparinized. Erythrocytes were washed and suspended as described (12) and the hematocrit was adjusted to 4.5%.

Determination of cytochrome c oxidase. The enzyme activity was measured spectrophotometrically (13). When the activity in mitochondria was determined, the changes of the absorbance of 550-540 nm was measured in a Hitachi 557 spectrophotometer (dual-wave length mode).

The activity of the purified and "reconstituted" enzyme was measured at 550 nm in a Beckman DU-7HS spectrophotometer. When necessary, the "log rate" of the reaction velocity was calculated with the built-in computer. The temperature of the cuvette holder was kept at 25 C with a circulating water bath (Lauda RM3 or RM6).

The reaction mixture (0.8 ml) contained 50 mM glycylglycine buffer (pH 7.4), 3.1 mM Tris-HCl (pH 7.4), 2 mM EDTA-2K, and 0.08% cytochrome c (reduced form). Lysophingolipid and galactosylceramide were dissolved first in ethanol and dimethyl sulfoxide, respectively, and added to the reaction mixture. The maximum concentration of ethanol and dimethyl sulfoxide in the reaction mixture was 2.5%, and any effects of the solvent were corrected (subtracted).

The protein concentration of the enzyme source (0.02 ml) was 1 or 2 mg/ml (mitochondrial suspension in 0.25 M sucrose with 5 mM Tris-HCl [pH 7.4]), and 0.27 mg/ml (purified cytochrome c oxidase) or 0.027 mg/ml ("reconstituted" cytochrome c oxidase). The protein was measured by the method of Lowry et al. (14).

The effects of albumin and γ-globulin on cytochrome c oxidase activity were examined by adding 0.08 ml of the 10% protein solution to the reaction mixture.

Hemolysis of erythrocytes. The isotonic buffer (30 mM Tris-HCl [pH 7.4] with 120 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$) (0.675 ml) and 0.025 ml of ethanol (with or without lysosphingolipid) or dimethyl sulfoxide (with or without galactosylceramide) were mixed. After the erythrocyte suspension (0.3 ml) in the isotonic buffer was added and incubated at 37 C for one hr with gentle shaking, 1.5 ml of the isotonic buffer was added and vortexed. After centrifugation at 1,000 × g for 15 min, the absorbance of the supernatant at 540 nm was measured. The percentage of hemolysis was calculated, taking the absorbance of the supernatant without lipid as 0% hemolysis and that of the supernatant obtained after incubation with water as 100% hemolysis.

The effects of albumin or γ-globulin on lysosphingolipid-induced hemolysis were examined by mixing 0.675 ml of the buffer (with or without protein), 0.3 ml of erythrocyte suspension and 0.025 ml of ethanol containing lysosphingolipid.

Binding of lysosphingolipids to proteins. Ethanol (0.025 ml) containing 6 mM lysosphingolipid and 0.695 ml of the buffer (30 mM Tris-HCl [pH 7.4] with 120 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$) were mixed, then 0.08 ml of the buffer without protein, or the buffer containing either 10% albumin or γ-globulin was added. An aliquot (0.7 ml) was placed in the Centrifree and centrifuged at 1,500 × g for 25 min at 25 C. Methanol (1.8 ml) and water (2.7 ml) were added to 0.6 ml of the filtrate. This mixture was applied twice to Sep-Pak C18 cartridge. After the cartridge was washed with 70 ml of water, the lipid was eluted with 5 ml of methanol and 10 ml of chloroform/methanol (2:1, v/v) and dried under a stream of nitrogen. The physical set-up and washing of the cartridge were done as described (15). The lipid was dissolved in 0.075 ml of chloroform/methanol (2:1, v/v) and 0.02 ml was applied to borate-impregnated HPTLC plate. After developed in chloroform/methanol/ammonia water (75:25:5, v/v/v) without paper-lining, the lipids were visualized by spraying with cupric acetate and heating (150 C) (16). The plate was scanned with a Shimadzu CS-930 TLC scanner at 370 nm.

RESULTS

Effects on cytochrome c oxidase. Galactosylsphingosine, glucosylsphingosine and sphingosine all inhibited cytochrome c oxidase in mitochondria (used within 6 hr after preparation). The potency to inhibit cytochrome c oxidase did not differ among the lysosphingolipids and 5 μM (0.1 μmol/mg mitochondrial protein) caused more than 50% inhibition. In contrast, galactosylceramide did not inhibit the enzyme, but it activated cytochrome c oxidase mildly at 25 μM or higher (Fig. 1).

The purified cytochrome c oxidase was not inhibited but activated by lysosphingolipids of 40 μM or less. The activation by sphingosine was less pronounced than that by galactosylsphingosine or glucosylsphingosine. In addition, at 75 μM or higher, sphingosine inhibited the activity, but galactosylsphingosine and glucosylsphingosine still activated the enzyme to some extent (Fig. 2A). Galactosylsphingosine activated the enzyme even at 200 μM.

Addition of sonicated phosphatidylcholine activated the purified cytochrome c oxidase more than 40-fold. Galactosylsphingosine, glucosylsphingosine and sphingosine showed a clear inhibitory effect on this activated enzyme preparation. The potency to inhibit the enzyme did not differ among the lysosphingolipids (Fig. 2B).

Hemolysis. Galactosylsphingosine, glucosylsphingosine and sphingosine all hemolyzed washed human erythrocytes, but galactosylceramide did not. The potency to hemolyze erythrocytes did not differ among the lysosphingolipids, and 50% hemolysis was caused by approximately 50 μM of the lysosphingolipids (Fig. 3).

Suppression of lysosphingolipid effects by albumin. The complete inhibition of cytochrome c oxidase (in mitochondria) either caused by galactosylsphingosine, glucosylsphingosine or sphingosine was completely and immediately abolished upon addition of 1% albumin but not by 1% γ-glubulin (Fig. 4).

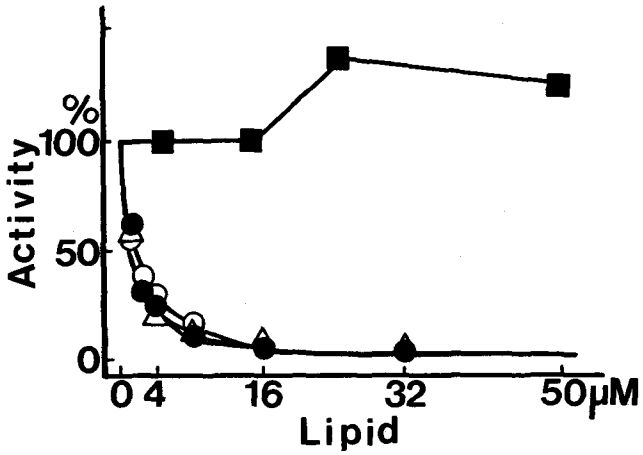

FIG. 1. The effects of galactosylsphingosine (●), glucosylsphingosine (○), sphingosine (△) and galactosylceramide (■) on cytochrome c oxidase activity in mitochondria. The reaction mixture (0.8 ml) contained 20 μg of mitochondrial protein.

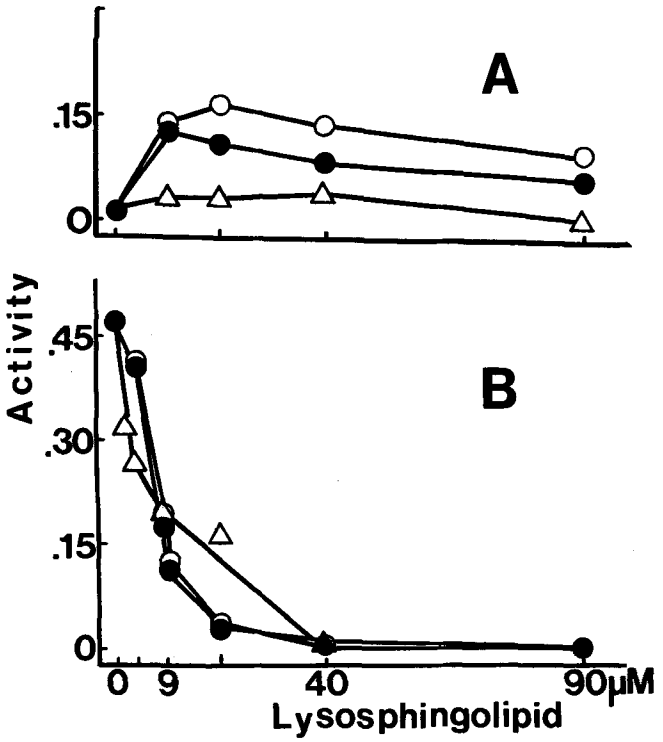

FIG. 2. The effects of galactosylsphingosine (●), glucosylsphingosine (○) and sphingosine (△) on the purified cytochrome c oxidase (A) and on the cytochrome c oxidase "reconstituted" with phosphatidylcholine (B). The reaction mixture (0.8 ml) contained 5.4 μg protein of cytochrome c oxidase in A and 0.54 μg protein in B. The activity (ordinate) is expressed in terms of arbitrary units ("log rate"/sec/mg protein).

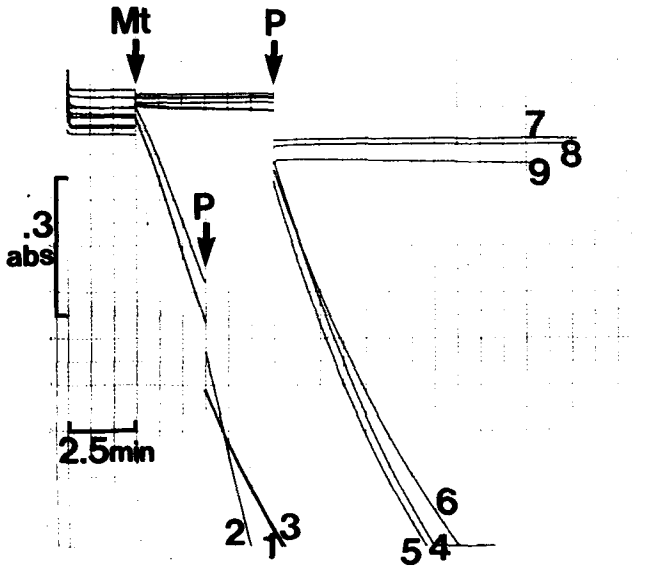

FIG. 4. The effects of albumin and γ-globulin on lysosphingolipid-induced inhibition of cytochrome c oxidase in mitochondria. The reaction was started by adding 0.02 ml of mitochondrial suspension (40 μg of protein) at Mt to the reaction mixture (0.78 ml). The speed of the decrease of the absorbance (550–540 nm) indicates the enzyme activity. No lysosphingolipid was present in 1, 2 and 3. At P, 0.08 ml of water was added to 1, 0.08 ml of 10% albumin (dissolved in water) to 2, and 0.08 ml of 10% γ-globulin (dissolved in water) to 3. One hundred μM galactoslysphingosine was present in 4 and 7, 100 μM glucosylsphingosine in 5 and 8, and 100 μM sphingosine in 6 and 9. After the cytochrome c oxidase activity was completely inhibited for five min, 0.08 ml of 10% albumin was added to 4, 5 and 6 at P, and 0.08 ml of 10% γ-globulin to 7, 8 and 9. The inhibition of the enzyme activity caused by lysosphingolipids was immediately abolished upon addition of albumin but not by γ-globulin.

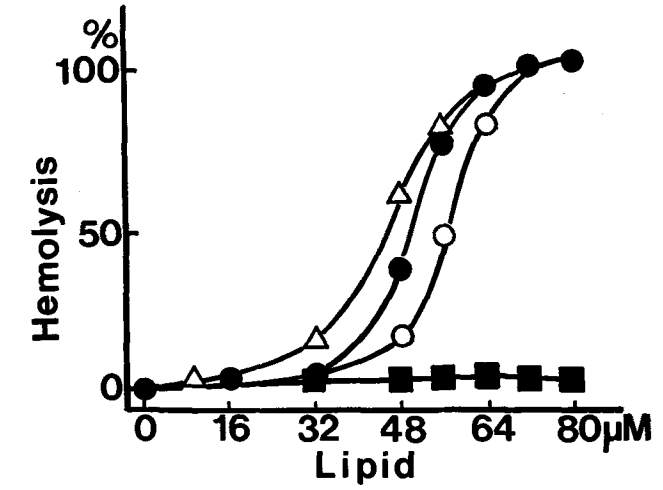

FIG. 3. Hemolysis caused by galactosylsphingosine (●), glucosylsphingosine (○), sphingosine (△) and galactosylceramide (■).

The 100% hemolysis, which occurred in the presence of 100 μM lysosphingolipids, also was completely prevented by 1–2% albumin but not by 1–4% γ-globulin (data not shown).

After mixing with γ-globulin and ultrafiltration, more than 50% of the galactosylsphingosine or glucosylsphingosine was recovered in the filtrate, but less than 3% of the lysosphingolipid was found in the filtrate when mixed with albumin (Fig. 5). Since solutions containing sphingosine did not filtrate well, binding of sphingosine to albumin could not be established by the present method.

DISCUSSION

Galactosylsphingosine, glucosylsphingosine and sphingosine all inhibited cytochrome c oxidase in mitochondria. When the enzyme was purified, however, these lysosphingolipids did not inhibit but did activate the enzyme to some extent. Cytochrome c oxidase is one of the typical membrane-bound enzymes (17), and its activity is very low in purified form. Hence, lipids, especially phospholipid(s), or a detergent is necessary to activate the purified enzyme (18). The present results show that even lysosphingolipids, which do not appear to be normal constituents of the mitochondrial membrane (19), can activate the enzyme to a limited extent. Phosphatidylcholine is one of the major lipids of mitochondria (19) and addition of sonicated phosphatidylcholine markedly activated the purified cytochrome c oxidase. The lysosphingolipids showed a clear inhibitory effect on this enzyme preparation, which suggests that the physiological lipid environment of the enzyme is necessary for lysosphingolipids to inhibit cytochrome c oxidase.

On the other hand, galactosylsphingosine and glucosylsphingosine caused hemolysis as reported by Taketomi and Nishimura (20) and by Taketomi and Yamakawa (21),

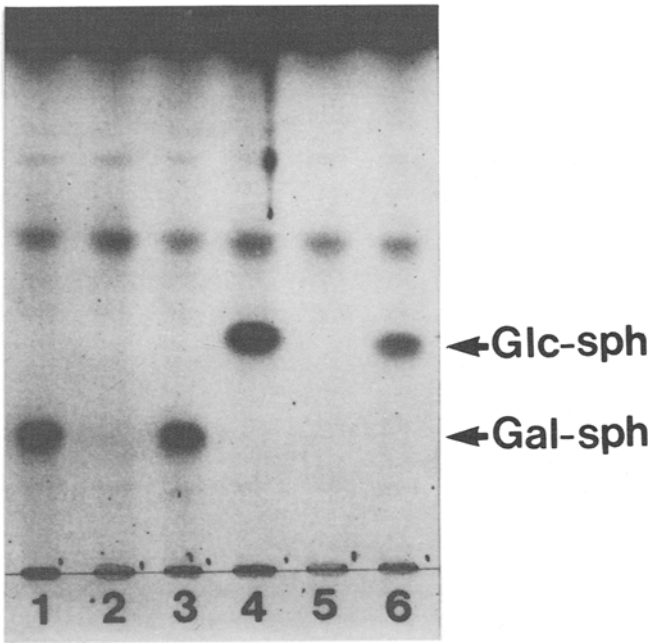

FIG. 5. Thin layer chromatogram of lipids recovered in the ultrafiltrate after mixing with no proteins (lanes 1 and 4), with albumin (lanes 2 and 5) and with γ-globulin (lanes 3 and 6). More than 50% of the control (lanes 1 and 4) of galactosylsphingosine (Gal-sph) or glucosylsphingosine (Glc-sph) are found in lanes 3 and 6 but less than 3% of the control in lanes 2 and 5.

respectively. In addition, we found that sphingosine also causes hemolysis. The mechanism of hemolysis is unclear, but obviously these lysosphingolipids can disrupt the erythrocyte membrane.

Taking these findings together, it seems possible that lysosphingolipids inhibit cytochrome c oxidase by perturbing its environment in mitochondrial membrane. The primary attacking site of lysosphingolipids may be the membrane structure of the cell, including mitochondria. However, it appears that not all membrane-bound enzymes are equally affected by lysosphingolipids. For instance, inhibition of NADH-cytochrome c reductase by galactosylsphingosine was much less than that of cytochrome c oxidase (8), and galactosylsphingosine showed no consistent effect on Na,K-ATPase of the erythrocyte membrane (Igisu et al., unpublished data). Thus, cytochrome c oxidase in mitochondria appears especially vulnerable to alteration of the membrane caused by lysosphingolipids.

The potency to hemolyze or to inhibit cytochrome c oxidase did not differ among galactosylsphingosine, glucosylsphingosine and sphingosine. This indicates that the sphingosine moiety plays an important role in the above effects. Moreover, since galactosylceramide showed neither effect, the free amino group in the sphingosine moiety appears responsible for either the inhibition of cytochrome c oxidase in mitochondria or the hemolysis.

The finding that albumin could counteract the effects of the lysosphingolipids may be of clinical relevance. Albumin is synthesized only in liver, and after being secreted it circulates in the bloodstream, but does not enter cell (22,23). Therefore, albumin cannot suppress the effects of lysosphingolipids within the cell, but the toxic effects of these lipids may not become apparent in blood because of the presence of serum albumin (>3 g/dl) (24).

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REFERENCES

1. Suzuki, K., and Suzuki, Y. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., and Brown, M.S., eds.) pp. 857–880, McGraw-Hill Book Company, New York.
2. Brady, R.O., and Barranger, J.A. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., and Brown, M.S., eds.) pp. 842–856, McGraw-Hill Book Company, New York.
3. Miyatake, T., and Suzuki, K. (1972) *Biochem. Biophys. Res. Commun. 48,* 538–543.
4. Kobayashi, T., Shinnoh, N., Goto, I., and Kuroiwa, Y. (1985) *J. Biol. Chem. 260,* 14982–14987.
5. Svennerholm, L., Vanier, M-T., and Mansson, J-E. (1980) *J. Lipid Res. 21,* 53–64.
6. Igisu, H., and Suzuki, K. (1984) *Science 224,* 753–755.
7. Nilsson, O., and Svennerholm, L. (1982) *J. Neurochem. 39,* 709–718.
8. Igisu, H., and Nakamura, M. (1986) *Biochem. Biophys. Res. Commun. 137,* 323–327.
9. Igisu, H., and Suzuki, K. (1984) *J. Neuropath. Exp. Neurol. 43,* 22–36.
10. Borgese, N., and Meldolesi, J. (1980) *J. Cell Biol. 85,* 501–515.
11. Merle, P., and Kadenbach, B. (1980) *Eur. J. Biochem. 105,* 499–507.
12. Igisu, H., and Mawatari, S. (1980) *Biochem. Med. 23,* 70–75.
13. Coruzzi, G., Trembath, M.K., and Tzagoloff, A. (1979) in *Methods in Enzymology* (Fleischer, S., and Packer, L., eds.) Vol. 56, pp. 95–106, Academic Press, New York.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
15. Igisu, H., and Suzuki, K. (1984) *J. Lipid Res. 25,* 1000–1006.
16. Fewster, M.E., Burns, B.J., and Mead, J.F. (1969) *J. Chromatogr. 43,* 120–126.
17. Tzagoloff, A. (1982) in *Mitochondria,* pp. 111–130, Plenum Press, New York.
18. Yu, C-A., Yu, L., and King, T.E. (1975) *J. Biol. Chem. 250,* 1383–1392.
19. Fleischer, S., Rouser, G., Fleischer, B., Casu, A., and Kirtchevsky, G. (1967) *J. Lipid Res. 8,* 170–180.
20. Taketomi, T., and Nishimura, K. (1964) *Jpn. J. Exp. Med. 34,* 255–265.
21. Taketomi, T., and Yamakawa, T. (1967) *Jpn. J. Exp. Med. 37,* 505–509.
22. Rothschild, M.A., and Oratz, M. (1976) in *Structure and Function of Plasma Proteins* (Allison, A.C., ed.) Vol. 2, pp. 79–105, Plenum Press, New York.
23. Birke, G., Liljedahl, S-T., and Rothschild, M. (1979) in *Plasma Proteins* (Blomback, B., and Hanson, L.A., eds.) pp. 54–71, John Wiley & Sons, Chichester, England.
24. Ganrot, P.O. (1972) *Scand. J. Clin. Lab. Invest. 29,* suppl. 124, 83–88.

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Triterpene Alcohols, 4-Methylsterols and 4-Desmethylsterols of Eggplant Seed Oil: a New Phytosterol

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4-Desmethylsterols, 4-methylsterols and triterpene alcohols of eggplant seed oil were isolated and characterized by ^1H nuclear magnetic resonance (NMR) and mass spectrometry. In addition to several compounds that have been shown to be present in eggplant seed oil, one triterpene alcohol and three sterols are described for the first time. One of these sterols, 14α-methyl-5α-cholest-9(11)-en-3β-ol, never before had been observed in plant lipids.
Lipids 23, 349–354 (1988).

This paper reports the study of the composition of 4-desmethylsterols, 4-methylsterols and triterpene alcohols of eggplant seeds (*Solanum melongena* L., Solanaceae). This plant is not used as a fat supply, and very few studies have been carried out on the sterolic and triterpenic composition of the lipid fractions of its seeds (1–5). Previous studies report only frequently found components, and our aim was to confirm prior investigations and to identify minor compounds. Since gas liquid chromatography (GLC) determination of relative retention time (RRT) of compounds is not sufficient proof of structure, a nonambiguous identification was performed by mass spectrometry (MS) and proton nuclear magnetic resonance (NMR); the latter technique required separation of pure components.

MATERIALS AND METHODS

Eggplant seeds were separated from the pulp of mature fruit by flotation in water and dried in an oven. They then were finely ground and Soxhlet extracted for 24 hr by hexane, producing a dark oil containing 1.8% of unsaponifiables after saponification with a 1 M solution of alcoholic potash. Thirty g of neutral alumina (E. Merck, Darmstadt, FRG) hydrated at 5% were used to chromatograph 1 g of the unsaponifiable fraction. The elution was carried out sequentially, first by pure hexane and then by hexane/diethyl ether mixtures of increasing polarity. The triterpene alcohols, eluted by a 80:20 mixture (v/v), represent 12% of the unsaponifiable lipids; the 4-methyl-sterols (22%) were eluted by a 70:30 mixture, and finally a 60:40 mixture separated the 4-desmethylsterols (30%). A GLC chromatogram of each fraction was taken (Delsi chromatograph equipped with a OV1701 capillary column [25 m long and 0.32 mm i.d.]; vector gas was helium [1 bar pressure], oven temperature was 270 C; oven temperature of injector and detector was 350 C). The RRT are expressed relative to cholesterol.

The triterpene alcohols (TA) were fractionated first by thin layer chromatography (TLC) on 0.2 mm-thick silica gel plates impregnated with silver nitrate (6,7). The elution was performed by four successive developments in CCl_4/CH_2Cl_2 (5:1, v/v). It must be pointed out that unlike the 4-desmethylsterols, acetylation is not necessary for good separation. The bands were visualized under UV light at 360 nm after spraying the plate with a 0.05% alcoholic solution of 2′,7′-dichlorofluorescein. The main bands were scraped off and extracted three times with hot chloroform. The resultant solution was filtered, evaporated, and the purity of the three fractions thus obtained was checked by GLC. The second and third fractions corresponded to pure compounds (**5h** and **5c**). The first fraction, which yielded two other pure components **4a** and **5a**, was analyzed by high performance liquid chromatography (HPLC) (Waters chromatograph equipped with a Merck Lichrosorb RP18 t μ column, 25 cm long and 4 mm i.d.; detection by refraction index (RI) variation; the mobile phase was anhydrous methanol at a flowrate of 0.8 ml/mn). The TA occurring in proportions less than 1.5% were not isolated. The four TA were studied by proton NMR and as tetramethylsilane (TMS) derivatives by mass spectrometry. The mass spectrometer was a Ribermag instrument (electron impact, 70 eV); the NMR instrument was a Bruker AC 360; spectra were taken in $CDCl_3$ solution with TMS as internal standard.

The 4-methylsterols (MS) were separated by semipreparative HPLC (Merck Lichrosorb RP18 7 μ column, 25 cm long and 10 mm i.d.; the mobile phase was anhydrous methanol with a flow rate of 4 ml/mn). Each fraction was purified by analytical HPLC; four major MS **3a, 3c, 3e** and **3f** were obtained pure. MS present in concentrations less than 2% were not isolated.

The 4-desmethylsterols (DS) first were separated by semipreparative HPLC; the five fractions thus obtained were analyzed by GLC and compared with the chromatogram of the whole DS fraction. The last two fractions were purified by analytical HPLC; the first three, which are mixtures, were acetylated overnight by acetic anhydride/pyridine at room temperature (6) and subjected to argentation TLC; elution was performed by six consecutive developments in CCl_4/CH_2Cl_2 (5:1, v/v). The major bands were scraped and then extracted by chloroform. The resultant solutions were filtered, evaporated, and the sterol acetates saponified. Purity was checked by GLC. All these procedures led to eight different sterols: **1a, 1b, 1c, 1d, 1e, 1f, 1g** and **2a**, which were studied by mass spectrometry and proton NMR. All DS representing more than 1% of the total sterolic fraction were isolated.

RESULTS

The isolated compounds were identified by analysis of the mass and proton NMR spectra, and by comparing the measured RRT with those reported in the literature (8,9).

The systematic study of the NMR spectra showed that seven of the eight isolated DS (**1a** to **1g**) possessed an intracyclic double bond in 5 (Scheme 1), according to the typical chemical shifts of H_6 and of the methyl groups

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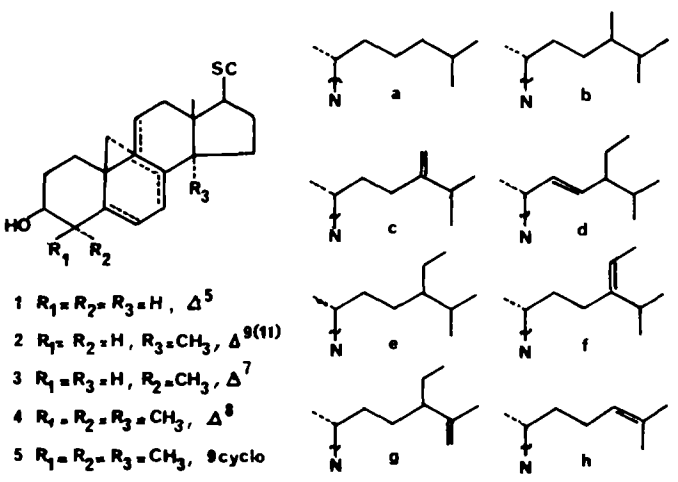
Abbreviations: DS, 4-desmethylsterols; TA, triterpene alcohols; MS, 4-methylsterols; TMS, tetramethylsilane; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; RI, refraction index; RRT, relative retention time; TLC, thin layer chromatography.



SCHEME 1

18 and 19 as shown in Table 1. This was confirmed by the mass spectra of the TMS derivatives of these DS (Table 2) leading, among others, to the ions of mass M-129 and 129 (10). The side chain of each of these seven DS was cleaved in mass spectrometry, with peak 255 (M^+-SC-TMSOH) always being observed; its formula was easily deduced from M^+-255+90. The location of the possible double bond was indicated in NMR by the chemical shifts and the signal multiplicity of the ethylenic protons. The configuration of C-24 was established, if necessary, according to the chemical shifts of methyl groups 21, 26, 27 and 28 (or 29) and compared with literature data (11-14): the methyl or ethyl substituent was α in every case (24*R* for **1b**, **1e** and 24*S* for **1d**), except for **1g** where it was β (24*S*); it corresponded to the biosynthesis of these structures as reported by Benveniste (28).

Sterol **2a** had a molecular mass of 472 (TMS derivative), which corresponded to a compound having 28 carbon atoms and a single double bond. The chemical shift of the intracyclic double bond ethylenic proton was 5.288, which did not correspond to a double bond in 5 (H-6: δ = 5.34 to 5.39) (15-16) or in 7 (H-7: δ = 5.14 to 5.16) (17,18). This led us to consider a double bond in 9 (11), rarely encountered in sterols but recently reported by Akihisa et al. (19), who observed a chemical shift of 5.29 for the H-11 proton of **2d**. Benveniste and Rahier (personal communication) found a chemical shift of 5.289 for the H-11 proton of synthetic 4α,14α-dimethyl-5α-ergost-9(11)-en-3β-ol acetate, while Gupta and Gupta (20) observed the corresponding signal at 5.35 in 5α-stigmasta-9(11),22-dien-3β-ol acetate. The cleavage of the side chain in mass spectroscopy (peaks 359 and 269) indicates a saturated side chain containing only eight carbon atoms, implying a 20-carbon atom nucleus. The NMR spectrum showed three singlets corresponding to nuclear methyl groups (δ = 0.661, 0.965, 0.750); the H_a-3 signal actually was a triplet of triplets, which excluded the structure of a 4-methylsterol, so the third methyl group must be in 14. It is pointed out that in mass spectrometry the M-Me peak is 100, which is characteristic of 14α-methylsterols (21). We thus suggest the 14α-methyl-5α-cholest-9(11)-en-3β-ol structure to take into account these observations. To our knowledge, this sterol has not yet been described

TABLE 1

^{1}H NMR Data (360 MHz) of Eggplant Seed 4-Desmethylsterols

	Chemical shifts (δ ppm)							
Protons	**1a**	**1b**	**1c**	**1d**[a]	**1e**	**1f**	**1g**[b]	**2a**[c]
CH_3 18 *(s)*	0.674	0.681	0.684	0.700	0.681	0.678	0.671	0.661
CH_3 19 *(s)*	1.002	1.008	1.011	1.012	1.009	1.003	1.010	0.965
CH_3 21 *(d)*	0.908	0.911	0.949	1.021	0.921	0.940	0.904	0.979
J (Hz)	6.8	6.4	6.3	7.0	6.6	6.6	6.4	6.1
CH_3 26 *(d)*	0.860	0.851	1.027	0.847	0.835	0.970	1.565	0.870
J (Hz)	6.8	6.8	6.9	6.4	7.3	7.2		6.8
CH_3 27 *(d)*	0.860	0.803	1.022	0.797	0.815	0.970		0.864
J (Hz)	6.8	6.3	6.9	6.7	7.3	7.2		6.5
H/CH_3 28		0.773	4.659/			5.097		
J (Hz)		6.4	(4.713)			6.7		
CH_3 29				0.806	0.845	1.581	0.801	
J (Hz)				7.4	7.7	6.7	7.3	
H 3 *(tt)*	3.514	3.519	3.515	3.518	3.524	3.512	3.516	3.586
J (Hz)	10.6	11.2	10.1	10.9	10.9	11.6	10.8	10.0
	4.7	5.6	4.3	4.7	4.3	4.9	4.2	5.2
H 6 *(d)*	5.345	5.347	5.353	5.343	5.349	5.340	5.346	
J (Hz)	5.1	5.1	4.7	4.7	5.4	5.2	4.9	
H 11 *(d)*								5.288
J (Hz)								5.9

[a]H 22,23: 5.022/5.164 (*dd*, *J* = 15.2, *J* = 8.5 Hz).

[b]CH_2 27: 4.636/4.723.

[c]CH_3 32: 0.750.

in a plant but has recently been reported by Goad et al. (22,23) in a sea animal (sea cucumber, *Psolus fabricii*). Our mass spectrometry (TMS derivative) and NMR (free DS) data are in good agreement with those reported by these authors, who studied acetates or free sterols. (The various spectral and chromatographic data are reported in Tables 1 and 2.)

On comparing our results with those in the literature (1,2) for eggplant seed 4-desmethylsterols, we confirm the presence of cholesterol **1a**, campesterol **1b**, stigmasterol **1d**, sitosterol **1e**, isofucosterol **1f**. The (24*S*) stigmasta-5,25-dien-3β-ol **1g**, and the 24-methylenecholesterol **1c** had not been reported previously in eggplant seed oil. The 14α-methylcholest-9(11)-en-3β-ol **2a** probably was confused with brassicasterol (1).

The four isolated 4-methylsterols have the nucleus of 4α-methyl-5α-cholest-7-en-3β-ol or lophenol according to the chemical shift values of methyl groups in 18 and 19, as well as that of H-7. The structure of the side chain was deduced from the mass spectra and from the NMR study of the ethylenic region (Tables 3 and 4). This was confirmed in mass spectrometry by intense fragments characteristic of Δ^7 sterols. In each case, we observed the typical peaks (24,25) $M^+ - SC$, $M^+ - SC - ROH$, $M^+ - SC - 2H - ROH$. The isolated compounds were 4α-methyl-5α-cholest-7-en-3β-ol (or lophenol, **3a**), 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol (or 24-methylenelophenol, **3c**), (24*R*) 4α-methyl-5α-stigmast-7-en-3β-ol (or 24*R*-ethyllophenol, **3e**) and (24*Z*) 4α-methyl-5α-stigmasta-7,24(28)-dien-3β-ol (or citrostadienol, **3f**). We could not isolate some minor MS as reported by Itoh et al. (3), among which was 4α,14α-dime-thylcholest-9(11)-en-3β-ol recorded at a trace level.

The four TA have a side chain of eight or nine carbon atoms according to their mass spectra (Table 5); three of them, **5a**, **5c** and **5h**, belong to the cycloartane series because of their specific cleavages in mass spectrometry ($M^+ - C_9H_{14} - TMSOH$) and of the characteristic signals of cyclopropanic protons in NMR (Table 6). These data and eventually the ethylenic proton patterns allowed an easy identification of cycloartanol (**5a**), cycloartenol (**5h**) and 24-methylenecycloartanol (**5c**). The authors who have studied NMR spectra of cycloartanol and its derivatives (e.g. 4,36–42) reported the same set of values for the chemical shifts of methyl groups 18, 30, 31, 32, but the correlations differ. The values we propose are based on the work by Iida and Kikuchi (43), who studied NMR spectra of **5a**, **5h** and **5c** in solutions containing increasing amounts of tri-(dipivalomethanato)europium, which allowed a nonambiguous proposition for methyl groups chemical shifts. These findings are confirmed by NMR data (19,44) relative to derivatives of pollinasterol (14α-methyl-9(19)cyclocholestan-3β-ol), which lacks 30 and 31 methyl groups. The fourth TA, **4a**, even though being

TABLE 2

Eggplant Seed 4-Desmethylsterol Data

Name	GLC RRT	HPLC RRT	Mass spectra of TMS derivatives and some diagnostic ions
Cholesterol **1a**	1.0	1.0	458 (12), 443 (6), 368 (40), 353 (23), 329 (54), 255 (13), 247 ($C_{18}H_{31}$, 12), 228 (5), 213 (12), 129 (100)
14α-Methyl-5α-cholest-9(11)-en-3β-ol **2a**	1.12	0.84	472 (41), 457 (100), 382 (2), 367 (77), 359 (M − SC, 3) 317 (M − cycle D − H, 4), 303 (M − cycle D − Me, 2), 291 (M − $C_{13}H_{25}$, 4), 269 (5), 241 (3), 227 (12), 213 (M − cycle D − Me − ROH, 12), 209 ($C_{15}H_{29}$, 10), 201 (291 − ROH, 14)
Campesterol **1b**	1.24	1.09	472 (16), 457 (9), 382 (39), 367 (20), 343 (58), 261 ($C_{19}H_{33}$, 12), 255 (17), 227 (6), 213 (13), 129 (100)
24-Methylene-cholesterol **1c**	1.24	0.94	470 (8), 455 (4), 386 (M − C_6H_{11} − H, 26), 380 (12), 371 (M − C_7H_{13} − 2H, 7), 365 (7), 343 (M − SC − 2H, 14), 341 (11), 296 (386 − ROH, 11), 281 (371 − ROH, 7), 255 (6), 253 (343 − ROH, 11), 228 (3), 213 (7), 129 (100)
Stigmasterol **1d**	1.32	1.00	484 (23), 394 (31), 379 (13), 355 (12), 351 (M − C_3H_7, 18), 283 (M − C_8H_{15}, 7), 255 (43), 253 (M − SC − 2H − ROH, 12), 228 (9), 213 (12), 129 (100)
Sitosterol **1e**	1.53	1.18	486 (13), 471 (7), 396 (30), 381 (14), 357 (46), 255 (10), 228 (2), 213 (12), 129 (100)
Stigmasta-5,25-dien-3β-ol **1g**	1.53	0.94	484 (26), 469 (4), 394 (19), 386 (M − C_7H_{14}, 5), 379 (9), 371 (M − C_8H_{17}, 5), 355 (11), 343 (M − SC − 2H, 7) 296 (386 − ROH, 2), 281 (371 − ROH, 3), 255 (6), 253 (343 − ROH, 8), 228 (3), 213 (7), 129 (100)
Isofucosterol **1f**	1.57	0.94	484 (8), 469 (6), 394 (10), 386 (M − C_7H_{14}, 84), 371 (11), 355 (10), 296 (386 − ROH, 65), 281 (M − C_8H_{17} − ROH, 45), 255 (11), 253 (M − SC − 2H − ROH, 10), 228 (7), 213 (13), 129 (100)

TABLE 3

Eggplant Seed 4-Methylsterol Data

Name	GLC RRT	Mass Spectra of TMS derivatives and some diagnostic ions
Lophenol **3a**	1.37	472 (100), 357 (13), 382 (8), 367 (12), 359 (4), 269 (31), 227 (12)
24-Methylenelophenol **3c**	1.68	484 (43), 569 (6), 400 (M − C_6H_{12}, 86), 394 (12), 379 (13), 357 (100), 310 (400 − ROH, 25), 267 (11), 242 (18), 227 (21)
24-Ethyllophenol **3e**	1.87	500 (100), 485 (12), 410 (7), 395 (9), 381 (M − Et − ROH, 2) 359 (M − SC, 5), 302 (3), 269 (M − SC − ROH, 40), 242 (3), 227 (16)
Citrostadienol **3f**	1.94	498 (8), 483 (6), 400 (M − C_7H_{13} − H, 86), 393 (4), 385 (M − C_8H_{15} − 2H, 5), 357 (M − SC − 2H, 100), 310 (400 − ROH, 6), 295 (385 − ROH, 8), 267 (357 − ROH, 13), 241 (9), 227 (11)

TABLE 4

^{1}H NMR Data (360 MHz) of Eggplant Seed 4α-Methylsterols

	Chemical shifts (δ ppm)			
Protons	**3a**	**3c**	**3e**	**3f**
CH_3 18 *(s)*	0.533	0.537	0.535	0.538
CH_3 19 *(s)*	0.827	0.826	0.827	0.828
CH_3 21 *(d)*	0.987	0.952	0.926	0.953
J (Hz)	6.3	6.4	6.5	6.5
CH_3 26,27	0.867/0.863	1.025/1.020	0.836/0.816	0.977
J (Hz)	6.7 6.5	6.9 6.8	6.1 7.6	7.2
CH_3 30 *(d)*	0.918	0.985	0.989	0.989
J (Hz)	5.8	6.4	6.5	6.3
H 3 *(dt)*	3.118	3.120	3.125	3.125
J (Hz)	10.6/4.7	10.1/4.3	10.9/4.3	10.8/4.2
H 7 *(d)*	5.177	5.182	5.184	5.188
J (Hz)	5.2	5.3	5.9	6.0
H 28		4.657/4.713		5.109
CH_3 29			0.845	1.590
J (Hz)			8.6	6.7

TABLE 5

Eggplant Seed Triterpene Alcohol Data

Name	GLC RRT	Mass spectra of TMS derivatives and some diagnostic ions
Dihydrolanosterol **4a**	1.37	500 (24), 485 (22), 395 (100), 297 (1), 273 (M − $C_{10}H_{17}$ − ROH, 2), 269 (M − $C_{10}H_{20}$ − H − ROH, 1.5), 255 (M − $C_{11}H_{22}$ − H − ROH, 4), 241 (M − $C_{11}H_{22}$ − Me − ROH, 7)
Cycloartanol **5a**	1.55	500 (4), 485 (7), 410 (90), 395 (62), 367 (M − C_3H_7 − ROH, 6), 341 (M − C_5H_9 − ROH, 44), 297 (13), 288 (M − C_9H_{14} − ROH, 23), 255 (M − $C_{11}H_{23}$ − ROH, 3), 241 (M − $C_{11}H_{22}$ − Me − ROH, 3)
Cycloartenol **5b**	1.73	498 (4), 483 (5), 408 (83), 393 (46), 365 (M − C_3H_7 − ROH, 58), 339 (M − C_5H_9 − ROH, 26), 297 (5), 286 (M − C_9H_{14} − ROH, 14), 255 (4), 241 (4)
24-Methylenecycloartanol **5c**	1.97	512 (2), 497 (3), 422 (38), 407 (19), 379 (31), 353 (8), 339 (2), 323 (2), 300 (12), 297 (8), 255 (3), 241 (3)

TABLE 6

^{1}H NMR Data (360 MHz) of Eggplant Seed Triterpene Alcohols

Protons	Chemical shifts (δ ppm)			
	4a	5a	5c[a]	5h[b]
CH_3 18 *(s)*	0.686	0.967	0.968	0.964
CH_3/CH_2 19	1.001	0.553/0.332	0.560/0.329	0.557/0.327
J (Hz)		4.4	4.3	3.9
CH_3 21 *(d)*	0.888	0.861	0.897	0.882
J (Hz)	6.7	6.6	6.5	5.9
CH_3 26,27	0.866	0.865/0.870	1.025/1.030	1.604/1.683
J (Hz)	6.6	6.6	6.9	
CH_3 30 *(s)*	0.982	0.962	0.968	0.965
CH_3 31 *(s)*	0.811	0.810	0.799	0.809
CH_3 32 *(s)*	0.877	0.892	0.900	0.882
H 3 *(dd)*	3.237	3.286	3.281	3.280
J (Hz)	11.3/4.4	10.8/4.2	10.3/4.2	9.8/3.9

[a]H 28: 4.663/4.716.

[b]H 24 (t): 5.101 (J = 6.8 Hz).

TABLE 7

Percent Composition of Sterols and Triterpene Alcohols of Eggplant Seed

4-Desmethylsterols:	47.0%	4-Methylsterols:	34.0%	Triterpene alcohols:	19.0%
Cholesterol	4.1	Lophenol	3.0	Dihydrolanosterol	2.4
14α-Methylcholest-9(11)-en-3β-ol	0.5	24-Methyleneloph.	2.3	Cycloartanol	9.9
Campesterol and 24-Methylenecholest.	5.2	24-Ethyllophen.	18.3	Cycloartenol	5.8
Stigmasterol	3.8	Citrostadienol	6.8	24-Methylene-cycloartanol	0.3
Sitosterol and stigmasta-5,25-dienol	30.3	Unknown	3.6	Unknown	0.6
Isofucosterol	3.0				
Unknown	0.1				

chemical shift values of the methyl groups 18, 19 and 32 are in agreement with a Δ^8 lanostane nucleus structure (35); **4a** is therefore the 24,25-dihydrolanosterol.

This study completes our knowledge of eggplant seed oil sterolic compounds, and Table 7 summarizes the qualitative and quantitative composition of this fraction. Components **2a**, **1c**, **1g** and **5c** never have been reported in that oil, and 14α-methyl-5α-cholest-9(11)-en-3β-ol **2a** been described among plant sterols; it belongs to 14α-methyl-$\Delta^{9(11)}$-sterol series, a member of which (**2e**) recently has been described by Akihisa et al. (19) in *Cucumis sativus* aerial parts.

The biosynthetic pathway seems to be usual (26–28) for the most abundant sterols. Note the presence of **4a**, a lanostane series compound. This type of skeleton frequently is found in botanical families such as Solanaceae (29); the origin of lanostane-type compounds is always a matter of controversy (30). The Δ^8 unsaturation can be produced either by a direct cyclization of squalene epoxyde, as it is the rule in nonphotosynthetic organisms, or by opening of the cyclopropane ring of cycloartenol **5h** or its derivatives; that pathway could be demonstrated in vivo (31) and even in vitro on Euphorbiaceae latex (32,33). Similar problems arise for the $\Delta^{9(11)}$ sterol **2a**, related to the triterpene parkeol (5α-lanosta-9(11),24-dien-3β-ol). If evolution of the side chain and the loss of methyl groups in 4 presumably are performed in the usual way, the $\Delta^{9(11)}$ double bond could have several origins. The first eventuality implies a migration of lanosterol (or a Δ^8 sterol) double bond towards the $\Delta^{9(11)}$ position; this is in contradiction with a very recent work by Akhila et al. (45), who studied the biosynthesis of 5α-stigmast-9(11)-en-3β-ol in *Costus speciosus* fed with labeled mevalonate. These authors observed that a tritium atom, coming from the carbon atom in 9, was found in the 8-position after the rearrangement, which excludes a Δ^8 intermediate. A second eventuality corresponds to a direct formation of parkeol from squalene epoxyde, the final rearrangement involving one more bond than in lanosterol biosynthesis in nonphotosynthetic species, but we could not find

parkeol in TA fraction of eggplant seed oil. A third eventuality is the opening of a 9,19 cyclopropane ring to give a $\Delta^{9(11)}$ sterol. Such a possibility has been shown to occur in vitro by action of a strong acid (34). A similar transformation could affect cycloartenol or its metabolites in the Solanaceae family (29).

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REFERENCES

1. Mannino, S., and Amelotti, G. (1975) *Riv. Ital. Sost. Grasse 52,* 79-83.
2. Itoh, T., Tamura, T., and Matsumoto, T. (1977) *Steroids 30,* 425-433.
3. Itoh, T., Ishii, T., Tamura, T., and Matsumoto, T. (1978) *Phytochemistry 17,* 971-977.
4. Itoh, T., Tamura, T., and Matsumoto, T. (1977) *Phytochemistry 16,* 1723-1726.
5. Itoh, T., Tamura, T., Sagawa, M., Tamura, T., and Matsumoto, T. (1980) *Phytochemistry 19,* 2491-2492.
6. Itoh, T., Sakurai, S., Tamura, T., and Matsumoto, T. (1980) *Lipids 15,* 22-25.
7. Itoh, T., Tamura, T., and Mastumoto, T. (1974) *Lipids 9,* 173-184.
8. Itoh, T., Tani, H., Fukushima, K., Tamura, T., and Matsumoto, T. (1982) *J. Chromatogr. 234,* 65-76.
9. Homberg, E. (1977) *J. Chromatogr. 139,* 77-84.
10. Brooks, C.J.W., Horning, E.C., and Young, J.S. (1968) *Lipids 3,* 391-402.
11. Nes, W.R., Krevitz, K., and Behzadan, S. (1976) *Lipids 11,* 118-126.
12. Rubinstein, I., Goad, L.J., Clague, A.D.H., and Mulheirn, L.J. (1976) *Phytochemistry 15,* 195-200.
13. Nes, W.R., Krevitz, K., Joseph, J., Nes, W.D., Harris, B., Gibbons, G.F., and Patterson, G.W. (1977) *Lipids 12,* 511-527.
14. Delseth, C., Kashman, Y., and Djerassi, C. (1979) *Helv. Chim. Acta 62,* 2037-2045.
15. Garg, V.K., and Nes, W.R. (1986) *Phytochemistry 25,* 2591-2597.
16. Akihisa, T., Tamura, T., and Matsumoto, T. (1987) *Phytochemistry 26,* 575-577.
17. Farines, M., Soulier, J., Charrouf, M., and Cavé, A. (1984) *Rev. Fr. Corps Gras 31,* 443-448.
18. Garg, V.K., and Nes, W.R. (1984) *Phytochemistry 23,* 2919-2923.
19. Akihisa, T., Shimizu, N., Tamura, T., and Matsumoto, T. (1986) *Lipids 21,* 491-493.
20. Gupta, G.S., and Gupta, N.L. (1974) *Tetrahedron Lett. 13,* 1221-1224.
21. Doyle, P.J., Patterson, G.W., Dutky, S.R., and Cohen, C.F. (1971) *Phytochemistry 10,* 2093-2095.
22. Goad, L.J., Garneau, F.X., Simard, L.J., ApSimon, J.W., and Girard, M. (1985) *Tetrahedron Lett. 26,* 3513-3516.
23. Goad, L.J., Garneau, F.X., Simard, J.L., ApSimon, J.W., and Girard, M. (1986) *Comp. Biochem. Physiol. 24B,* 189-196.
24. Zaretskii, Z.C. (1976) in *Mass Spectrometry of Steroids,* p. 107, Wiley, New York.
25. Dumazer, M., Farines, M., and Soulier, J. (1986) *Rev. Fr. Corps Gras 33,* 151-156.
26. Goad, L.J., and Goodwin, T.W. (1972) in *Progress in Phytochemistry* (Reinhold, L., and Liwschitz, Y., eds.) Vol. 3, pp. 113-198, Wiley, New York.
27. Nes, W.R. (1977) in *Advances in Lipid Research* (Paoletti, R., and Kritchevsky, D., eds.) Vol. 15, pp. 233-324, Academic Press, New York.
28. Benveniste, P. (1986) *Ann. Rev. Plant Physiol. 37,* 275-308.
29. Heftmann, E. (1983) *Phytochemistry 22,* 1843-1860.
30. Nes, W.R., and Nes, W.D. (1980) in *Lipids in Evolution* (Kritchevsky, D., ed.), pp. 157-184, Plenum Press, New York and London.
31. Rahier, A., Cattel, L., and Benveniste, P. (1977) *Phytochemistry 16,* 1187-1192.
32. Ponsinet, G., and Ourisson, G. (1967) *Phytochemistry 6,* 1235-1243.
33. Ponsinet, G., and Ourisson, G. (1968) *Phytochemistry 7,* 757-764.
34. Shimizu, N., Itoh, T., Saito, M., and Matsumoto, T. (1984) *J. Org. Chem. 49,* 709-712.
35. Sekula, B.C., and Nes, W.R. (1980) *Phytochemistry 19,* 1509-1512.
36. Salt, T.A., and Adler, J.H. (1985) *Lipids 20,* 594-601.
37. Heupel, R.C. (1985) *Phytochemistry 24,* 2929-2937.
38. Laonigro, G. Adinolfi, M., Barone, G., Lanzetta, R., and Mangoni, L. (1982) *Gaz. Chim. Ital. 112,* 273-275.
39. Salt, T.A., and Adler, J.H. (1986) *Lipids 21,* 754-758.
40. Iida, T., Tamura, T., and Matsumoto, T. (1980) *J. Lipid Res. 21,* 326-338.
41. Itoh, T., Tamura, T., and Matsumoto, T. (1976) *Steroids 27,* 275-285.
42. De Pascual Teresa, J., Urones, J.G., Marcos, I.S., Basabe, P., Sexmero Cuadrado, M.J., and Fernandez Moro, R. (1987) *Phytochemistry 26,* 1767-1776.
43. Iida, T., and Kikuchi, M. (1977) *Chem. Phys. Lipids 20,* 157-174.
44. Akihisa, T., Shimizu, N., Tamura, T., and Matsumoto, T. (1986) *Lipids 21,* 494-497.
45. Akhila, A., Gupta, M.M., and Thakur, R.S. (1987) *Tetrahedron Lett. 28,* 4085-4088.

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METHODS

A New Quantitative Method for the Analysis of Monoacylglycerol Isomers Using ^{13}C Nuclear Magnetic Resonance Spectroscopy[1]

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Current methods for determining the regiochemistry of monoacylglycerols are lengthy, tedious and aggravated by the ready isomerization of 2-acyl-*sn*-glycerols. A new method employs a very rapid adsorption chromatography step in which isomerization is kept to a minimum, followed by formation of the isopropylidene derivatives using mild procedures. These cyclic derivatives of 1- and 3-acyl-*sn*-glycerols and 2-acyl-*sn*-glycerols are stable thermally and display certain characteristic ^{13}C NMR resonances. Integration of these resonances yields directly the proportion of monoacylglycerol isomers in the mixture. The method was applied to the analysis of monoacylglycerols produced by enzymatic hydrolysis of synthetic and naturally occurring triacylglycerols.
Lipids 23, 355–358 (1988).

Fatty acid monoesters of glycerol can differ in the location of the fatty acid residue (1- or 3-acyl-*sn*-glycerols or 2-acyl-*sn*-glycerol) and in the chain length and degree of unsaturation of the fatty acid residues. For the purpose of the following discussion, the two regiochemically distinct species will be referred to as 1- and 2-monoacylglycerols, respectively. Such compounds, together with diacylglycerols, find wide use in the food industry to facilitate more homogeneous and more easily processed foods. In biology, monoacylglycerols are important intermediates and products of many lipid reactions. Characterization of monoacylglycerols, therefore, is important for process control in industry and in defining their role and distribution in the cell.

A number of methods have been developed to determine the proportions of 1- and 2-monoacylglycerols in a mixture including periodate oxidation (1), thin layer chromatography (TLC) on boric acid-impregnated silica gel (2,3), gas liquid partition chromatography (GLC) of tetramethylsilyl derivatives (3–5) and high pressure liquid chromatography (HPLC) (6–8), which has not yet fulfilled its obvious potential. However, the periodate method is tedious and prone to errors due to isomerization through intramolecular acyl migration (9), quantitation by TLC has been questioned (6), and the GLC method proved more sensitive to the chain length of the acyl chain than its position on glycerol (4). Conversion of monoacylglycerols to allyl esters, before and after periodate oxidation, apparently provided quantitative information not only on monoacylglycerol composition but also the fatty acid distribution in each type of monoacylglycerol (10).

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Abbreviations: AT, acquisition time; DMP, 2,2-dimethoxypropane; FA, flip angle; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; PPTA, pyradinium *p*-toluenesulfonate; RT, repetition time; SW, sweep width; TLC, thin layer chromatography.

The purpose of this communication is to report an alternative quantitative method of determining the proportions of 1- and 2-monoacylglycerols by converting them to five- and six-membered cyclic isopropylidene derivatives, respectively, and subsequently identifying and estimating their proportions through integration of the characteristic quaternary acetal carbon resonances in the ^{13}C NMR spectrum. This nondestructive method has additional advantages that accrue from a mild derivatization procedure that precludes intramolecular acyl migration in the monoacylglycerols, and the insensitivity of the quaternary acetal carbon resonances to the nature of the acyl residues in the derivatives.

MATERIALS AND METHODS

Materials. Pancreatin (Grade II, porcine pancreas), monopalmitoylglycerol, trilinolenoylglycerol and tricaproylglycerol were obtained from Sigma Chemical Co. (St. Louis, MO). Peanut oil was purchased from BDH Chemicals (Toronto, Ont.), and olive oil and coconut oil were obtained as commercial food-grade oils.

Instrumentation. GLC analyses were performed using a Varian 3400 operating in the FID mode and equipped with a fused silica capillary column (30 m × 0.25 mm) liquid phase DB-5, carrier gas He, flow rate 3 ml/min, injection port 310 C, detector 310 C, operation was programmed from 150–270 C at 5 C/min. All samples for NMR analyses were dissolved in $CDCL_3$ using TMS as internal standard. Pulse Fourier Transform ^1H NMR spectra were recorded at 300 MHz at ambient temperature using a Bruker model MSL 300 spectrometer, sweep width (SW) 4504 Hz, data size 16384 Hz, acquisition time (AT) 1.82 s flip angle (FA) 90°, repetition time (RT) 6.8 sec. ^{13}C NMR spectra were recorded either at 20 MHz at ambient temperature using a Varian FT 80A spectrometer, SW 4132 Hz, AT 0.99 s, FA 40°, pulse delay (PD)0 s, ^1H broadband decoupling, or at 75.4 MHz using a Bruker model MSL 300; spectral width 16.7 KHz, data size 16 K zero-filled to 32 K [1.00 Hz/pt.], 30° pulse, (RT) 1 s, ^1H broadband decoupling, 5 mm tubes. Some spectra also were recorded with samples containing chromium (III) acetylacetonate (5 mg/ml), PD 2 s, and suppression of Nuclear Overhauser Enhancement (NOE) for comparison of ^{13}C intensities.

Mass spectra were obtained with a DuPont model 21-110B double-focusing spectrometer used in the electrical detection mode, 8 kV accelerating voltage, 70 eV ionizing energy. Accurate mass measurements were made by peak matching against a perfluorokerosene reference.

Enzymatic hydrolysis. Triacylglycerol (0.5 g) was suspended in a mixture of tris (hydroxymethyl) methylamine buffer (6 ml, 0.5 M, pH 7.5), aqueous $CaCl_2$ (0.5 ml, 45% w/v) and aqueous sodium deoxycholate (2 ml, 1% w/v). Pancreatin (0.5 g) was suspended in tris

(hydroxymethyl) methylamine buffer (3 ml, 0.5 M, pH 8.0). The suspensions were warmed to 40 C, combined, and agitated for 10 min. The aqueous suspension was extracted with dichloromethane (2 × 25 ml), washed with distilled water, dried (anhydrous Na_2SO_4) and evaporated to dryness. The residue was dissolved in diethyl ether/hexane (10:90, v/v), applied to a short silica gel (Mallinckrodt silicAR CC-7, 200–325 mesh) column (3 cm × 4.5 cm), and eluted under vacuum with portions (100 ml) of (a) diethyl ether/hexane (20:80, v/v), (b) diethyl ether/hexane (50:50, v/v) and (c) diethyl ether. Fractions eluted with solvents (a), (b) and (c) contained fatty acids and triacylglycerols, diacylglycerols and monoacylglycerols, respectively. Fractions were monitored by TLC (Merck pre-coated sheets, silica gel 60F-254: eluant ethyl acetate/hexane [50:50, v/v]). Evaporation of the diethyl ether fraction gave pure monoacylglycerol.

Isopropylidene formation. The monoacylglycerol (0.13 g) was dissolved in a mixture of dichloromethane (2 ml) and 2,2-dimethoxypropane (2 ml) containing pyridinium *p*-toluenesulfonate (0.01 g). The reaction mixture was maintained at ca. 35 C for two hr and then worked up by either method A or B. Method A: the reaction mixture was diluted with dichloromethane (30 ml), washed with aqueous $NaHCO_3$ (15 ml, 5%), dried (anhydrous Na_2SO_4) and evaporated to yield the monoacylglycerol isopropylidene derivative (yield 95%). Method B: solid $NaHCO_3$ (ca. 0.5 g) was added to the reaction mixture, which was stirred (10 min) and then evaporated to dryness. The residue was extracted with hexane (30 ml), filtered and evaporated to yield the monoacylglycerol isopropylidene derivative (yield 95%).

2,2-dimethyl-1,3-dioxan-5-yl hexanoate. Tricaproylglycerol (0.3 g) obtained from Sigma Chemical Co. was hydrolyzed using porcine pancreatin, and the monoacylglycerol product (0.094 g) was treated with DMP and traces of PPTS as described above to yield the dioxane (0.108 g, 95%); υ_{max}(neat) 1735, 1375, 1172, 830 cm^{-1}; ^1H NMR ($CDCl_3$). Δ_H 0.90 (3H, t, J = 6.6 Hz), 1.32 (6H, m), 1.44 (6H, s), 1.65 (2H, m), 2.38 (2H, t, J = 7.5 Hz), 3.81 (2H, dd, J = 3.3 and 12.8 Hz) 4.09 (2H, dd, J = 3.3 and 12.8 Hz), 4.70 (1H, t, J = 3.3 Hz); ^{13}C NMR ($CDCl_3$)δ_cc 13.88(q, C-6′), 22.34(t, C-5′), 24.67(t, C-4′), 25.77(q, acetal Me), 31.34(t, C-3′), 34.29(t, C-2′), 62.04(t, glycerol CH_2), 66.07(d, glycerol CH), 98.45 (s, acetal C), 173.40 (s, carbonyl); MS, M/Z 230 (M+, not observed), 215, 173, 99.

RESULTS AND DISCUSSION

1-Monopalmitoylglycerol was obtained commercially, and 2-monoacylglycerols were obtained by hydrolysis of natural or synthetic triacylglycerols with porcine pancreatin (11,12). Monoacylglycerols were isolated from the hydrolysis mixture in 10 min or less following rapid chromatography on a short dry-packed pad of silica gel. Thus, exposure of the labile 2-monoacylglycerols to silica gel was kept to a minimum.

2,2-Dimethoxypropane (DMP) has been used to make isopropylidene derivatives of 1,2- and 1,3-diols under very mild conditions and in high yields (13). This reagent readily derivatizes 2-monoacylglycerols, in contrast to acid/acetone, which promotes acyl migration rather than formation of the isopropylidene derivative (10,14). In this approach, DMP in the presence of a catalytic amount of pyridinium *p*-toluenesulfonate (PPTS) (15,16) or *p*-toluenesulfonic acid, rapidly (ca. two hr) and cleanly converts 1-monoacylglycerols 1 and 2-monoacylglycerols 2 to the respective isopropylidene derivatives 3 and 4 (Fig. 1). These conditions do not promote isomerization, even after prolonged reaction times (e.g. eight hr). To our knowledge, 2,2-dimethyl-1,3-dioxanes 4 have not been reported previously, and representative physical data are reported in Materials and Methods.

During the course of the reaction, intermediates were observed by TLC, especially when a 2-monoacylglycerol was the substrate. ^{13}C and ^1H NMR showed these intermediates to be the expected acyclic intermediates 5 and 6 in this stepwise acetal-exchange reaction: the resonances δ_c 48.46 and δ_H 4.20 ppm were characteristic of a methoxyl group and the resonance at δ_c 100.36 ppm was assigned to the quaternary carbon of an acyclic acetal. These intermediates were converted to the cyclic dioxolane or dioxane products under the reaction conditions. The addition of a small amount of *p*-toluenesulfonic acid increased the rate of this conversion without affecting the nature of the products.

Once formed, the isopropylidene derivatives 3 and 4 freeze the distribution of monoacylglycerol isomers in the mixture. These derivatives also are quite stable at room temperature and can be readily analyzed by ^{13}C NMR spectroscopy (Fig. 2). The quaternary dioxolane or dioxane carbons resonate at very different frequencies in an area of the spectrum that is virtually unique to this type of carbon, i.e. 95–115 ppm. The resonance frequency of the quaternary carbon of the dioxolane 3 (δ_c 109 ppm) and of the dioxane 4 (δ_c 98 ppm) are more than 10 ppm apart (17,18), and their chemical shift is independent of fatty acid chain length. Thus, the ratio of 3 to 4 can be determined directly by integration of these two signals. It also is worth pointing out that in these derivatives the glycerol carbons of 3 (δ_c 64.59, 66.56 and 73.84 ppm) and 4 (δ_c 62.04 and 66.07 ppm) are sufficiently distinct to be useful as an additional check on the quantitative results. For example, analysis of monolinolenoylglycerol, produced by enzymatic hydrolysis of trilinolenoylglycerol, gave the following results (% of 2-monoacylglycerol): 78% (acetal carbon), 76% (C-1 and C-3), 76% (C-2), average 76%. This compares with a value of 75% determined by GC analysis. The results of enzymatic hydrolysis of other triacylglycerols are shown in Table 1.

Under the GLC conditions described here, the derivatized monoacylglycerols appeared as a pair of peaks in

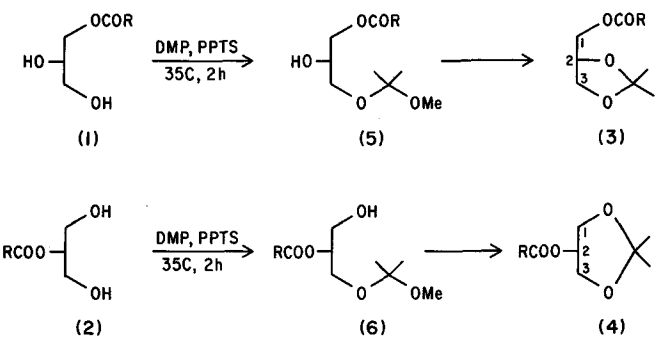

FIG. 1.

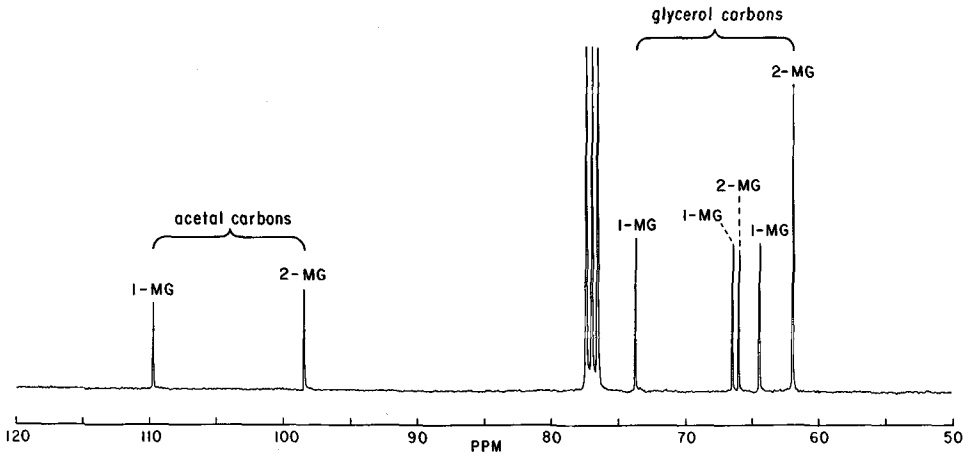

FIG. 2.

TABLE 1

Percentage of 2-acyl-*sn*-glycerol Produced by Deacylation of Synthetic and Natural Triglycerides with Porcine Pancreatin[a]

Triacylglycerol	Monoacylglycerol	% 2-acyl-*sn*-glycerol[c]	Reported[b]
	1(3)-monopalmitin (Sigma)	0[d]	
Olive oil	by enzymatic hydrolysis	≤97[d] (94)	84
Coconut oil	by enzymatic hydrolysis	88 (90)	88
Peanut oil	by enzymatic hydrolysis	86	—
Tricaproylglycerol	by enzymatic hydrolysis	92 (91)	63
	by enzymatic hydrolysis (30 min)	88 (82)	—
	by enzymatic hydrolysis and adsorption on silica (60 min)	74 (77)	—
Trilinolenoylglycerol	by enzymatic hydrolysis	75	59

[a]Each hydrolysis was performed as described in Materials and Methods. Data are the result of at least two separate experiments.

[b]Proportion of monoacylglycerol isomers determined by the periodate method (11,12).

[c]Ratios calculated from spectra recorded using standard conditions for proton noise decoupled (PND) spectra. The use of relaxation reagents [Cr(acac)$_4$] and prolonged delay times, yielding normalized spectra gave the same values. The values in parentheses correspond to the ratios determined by GLC analysis. The ratio of 1-acyl-*rac*-glycerol to 2-acyl-*sn*-glycerol isopropylidene derivatives in the oils was calculated by comparing the total integrated area of peaks corresponding to each isomer.

[d]Other isomer below detection limits.

which the 1-monoacylglycerol derivative is eluted before the 2-monoacylglycerol derivative. In a (complex) mixture like coconut oil or olive oil, these isopropylidene derivatives were eluted as a series of pairs of peaks, each pair corresponding to the nature and chain length of the attached fatty acid. The proportion of 1- and 2-monoacylglycerol derivatives in the mixture was calculated by comparing the total integrated area of peaks corresponding to each isomer. It is interesting to note that within error, the ratio of peaks for each pair in the mixture appears to be the same.

In all cases, the relative amounts of 2-monoacylglycerol determined by the ^{13}C method were equal to or greater than those determined by the periodate method (11,12) (see Table 1). It generally is assumed that most 1-monoacylglycerol arises from isomerization of 2-monoacylglycerol during the enzymatic hydrolysis or subsequent work-up (19). Since the hydrolysis used here was performed essentially as described, it appears that this method permits less isomerization during work-up and provides a more accurate representation of the ratio of 2- to 1-monoacylglycerols produced by enzymatic hydrolysis. For example, the improved yields of 2-monolinolenoylglycerol and 2-monocaproylglycerol are particularly noteworthy, as previous results were interpreted to mean that these compounds are particularly susceptable to isomerisation (19). In fact, in this NMR method, there is almost no isomerization of the 2-monoacylglycerol

following hydrolysis of tricaproylglycerol (Table 1). The lack of isomerization most likely is due to the rapid chromatography step and the mild conversion to the isopropylidene derivative, which is completely resistant to isomerization even at room temperature.

A higher proportion of 1-monoacylglycerol also was formed after hydrolysis of coconut and peanut oil. This was not due to the presence of 1-monoacylglycerol in the original mixture since treatment of the natural oil with DMP/PPTS yielded a product displaying none of the characteristic NMR signals associated with the isopropylidene derivatives. In the case of tricaproylglycerol, increasing enzyme reaction time to 30 min or prolonged treatment with DMP/PPTS had little effect on the monoacylglycerol ratio. It is more likely that isomerization occurs during the chromatography step and prolonged exposure (one hr) of underivatized 2-monocaproylglycerol to silica gel did increase the amount of 1-monoacylglycerol. However, if necessary the chromatography step can be eliminated, the hydrolysis products derivatized directly, and the mixture analyzed in the usual manner.

This nondestructive ^{13}C NMR method utilizing the stable isopropylidene derivatives is a useful and convenient tool for analysis of monoacylglycerol mixtures. The simplicity of the sample handling combined with the reliability of the results make it an attractive alternative to current methods of analysis. The further utility of these isopropylidene derivatives in GLC and GLC/MS analysis is also under investigation and shows some promise since the 1- and 2-monoacylglycerol derivatives display different mass spectral fragmentation pathways.

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REFERENCES

1. Official and Tentative Methods of the American Oil Chemists' Society, Vols. I and II, 3rd edn., American Oil Chemists' Society, Champaign, IL, 1966, Method Cd 11-57.
2. Thomas, A.F., Scharoun, J.E., and Ralston, H. (1965) *J. Am. Oil Chem. Soc. 42,* 789-792.
3. Mangold, H.K., ed. (1984) *CRC Handbook of Chromatography: Lipids,* Vol. I, pp. 381-409, CRC Press, Boca Raton, FL.
4. Itabashi, Y., and Takagi, T. (1979) *Lipids 15,* 205-215.
5. Soe, J.B. (1983) *Fette Seifen Anstrichm. 85,* 72-76.
6. Ritchie, A.S., and Jee, M.H. (1985) *J. Chromatogr. 329,* 237-280.
7. Riisom, T., and Hoffmeyer, L. (1978) *J. Am. Oil Chem. Soc. 55,* 649-652.
8. Hammond, E.W. (1981) *J. Chromatogr. 203,* 397-403.
9. Mattson, F.H., and Volpenhein, R.A. (1962) *J. Lipid Res. 3,* 281-296.
10. McInnes, A.G., Tattrie, N.H., and Kates, M. (1960) *J. Am. Oil Chem. Soc. 37,* 7-11.
11. Hirayama, O. (1964) *Agr. Biol. Chem. 28,* 193-200.
12. Hirayama, O., and Nakae, T. (1964) *Agr. Biol. Chem. 28,* 201-205.
13. Liptak, A., Imre, J., and Nanasi, P. (1981) *Carbohydr. Res. 92,* 154-156.
14. Martin, J.B. (1953) *J. Am. Chem. Soc. 75,* 5483-5486.
15. Miyashita, M., Yoshikoshi, A., and Grieco, P.A. (1977) *J. Org. Chem. 42,* 3772-3774.
16. Sterzycki, R. (1979) *Synthesis,* 724-725.
17. Buchanan, J.G., Chacon-Fuertes, M.E., Edgar, A.R., Moorhouse, S.J., Rawson, D.I., and Wightman, R.H. (1981) *Tetrahedron Lett.,* 1973-1976.
18. Buchanan, J.G., Edgar, A.R., Rawson, D.I., Shahidi, P., and Whightman, R.H. (1982) *Carbohydr. Res. 100,* 75-86.
19. Litchfield, C. (1972) in *Analyses of Triglycerides,* 1st edn., pp. 179-187, Academic Press, New York.

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Separation of α-Tocopherol and Its Oxidation Products by High Performance Liquid Chromatography

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A very sensitive high performance liquid chromatographic (HPLC) method was developed for the separation of α-tocopherol (α-T) and its five oxidation products: α-tocopheryl quinone (TQ), dimer (D), dihydroxy dimer (DHD), trimer (T) and 9-methoxy-α-tocopherone commonly called α-tocopheroxide (TO). The separation was achieved on a normal-phase silica-based column (Ultrasphere-Si), using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) at a flow rate of 0.4 ml/min, and the eluants were monitored simultaneously at their maximum absorptions using a variable-wavelength UV detector. The minimum detection limit is 0.01 μg for α-T, TQ and TO, 0.05 μg for DHD and D, and 0.1 μg for T/injection. This normal-phase method has the combined advantages of being very sensitive, fast and capable of separating all six compounds at the same time.
Lipids 23, 359-361 (1988).

α-Tocopherol (α-T), a naturally occurring antioxidant, reacts with free radicals or oxidizing agents to form several oxidation products. Some of these oxidation products include α-tocopheryl quinone (TQ), and dihydroxy dimer (DHD), dimer (D) and trimer (T) of α-tocopherol and 9-methoxy-α-tocopherone commonly called α-tocopheroxide (TO) (1-6). The first four of these oxidation products have been isolated as metabolites, and TQ was found to be the major metabolite (1,3,7).

Quantitative determination of α-T and its oxidation products (TOP) can be used to measure the degree of cell membrane peroxidation and the metabolic pathways of α-T, and useful in the prediction of the stability of oils (7,8). Gas chromatography, column chromatography and thin layer chromatography were methods used commonly for the separation of α-T and TOP (9-12). These methods have serious limitations because of their comparatively low sensitivity, long analysis time and possibility of increased oxidation. Lately, various high performance liquid chromatographic (HPLC) methods have been applied successfully to determine α-T in foods and feeds (13,14), and in tissues (15,16). HPLC methods also were reported for the simultaneous separation of α-T, TQ or TO (17-21) and for the separation of dimers of α-T (22).

Currently, a HPLC method is not available in the literature for the simultaneous separation of α-T and its five oxidation products (TQ, DHD, D, T and TO) with a very high sensitivity.

The present method is not only capable of simultaneously separating α-T and its most important five oxidation products in a single injection, but it has higher sensitivity than the previously published methods.

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Abbreviations: α-T, α-tocopherol; AUFS, absorption unit full scale; D, dimer; DHD, dihydroxy dimer; HPLC, high performance liquid chromatography; T, trimer; TO, α-tocopheroxide; TOP, α-T oxidation products; TQ, α-tocopheryl quinone.

MATERIALS AND METHODS

Reagents. α-T was purchased from Eastman-Kodak Company (Rochester, NY). Hexane (HPLC grade), methylene chloride (HPLC grade), methanol (ACS grade), ethanol (ACS grade) and diethyl ether (ACS grade) were purchased from Fisher Scientific Co. (Itasca, IL). Aluminum oxide was purchased from Bio-Rad Laboratory (Richmond, CA). All other chemicals used were reagent grade.

Synthesis and purification of standard TOP. The TQ was synthesized by the method of Eggitt and Norris (23), DHD, D and T according to Csallany et al. (3) and TO by the modified method of Boyer (24). Modification consisted of a change from ethanol to methanol in the synthesis of the compound. The TQ, DHD, D and T were purified by column chromatography on aluminum oxide, deactivated with 6% water, followed by HPLC on a 250 mm × 10 mm (i.d.) C_{18} reversed-phase semipreparatory column packed with Ultrasil-ODS (10 μm) (Beckman Instruments, Inc., Berkeley, CA). Purification of TO was carried out only on the above-mentioned reversed-phase C_{18} column. Standard stock solutions were made weight by volume. The HPLC was performed with a mobile phase of methanol/hexane/methylene chloride (95:4:1, v/v/v) at a flow rate of 0.4 ml/min for the purification of TQ and TO, and a mobile phase of methanol /hexane/methylene chloride (30:21:1, v/v/v) at a flow rate of 1.5 ml/min for the purification of D, DHD and T. The absorption spectrum of each purified compound was monitored by a DU-8 Spectrophotometer (Beckman Instruments, Inc.), using a quartz cuvette (1 × 1 cm).

HPLC equipment and conditions. The HPLC equipment for the separation of α-T and TOP consisted of a model 110A solvent metering pump (Beckman Instruments, Inc.) and an Altex Model 210 solvent injector equipped with a 100-μl loop (Beckman Instruments, Inc.). A normal-phase HPLC chromatography was performed on a 6-μm Ultrasphere-Si, 250 mm × 4.6 mm (i.d.) column, using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v), at a flow rate of 0.4 ml/min to separate α-T, TQ, DHD, D, T and TO. The UV absorptions were monitored at 292, 268, 292, 294, 298 and 240 nm for α-T, TQ, DHD, T, D and TO, respectively, using a Beckman model 165 variable-wavelength detector (with two channels) equipped with a 20-μl quartz flow cell (Beckman Instruments, Inc.). The wavelength was changed from 295 nm to 292 nm in the first channel after eluting T, D and DHD in order to monitor α-T at their maximum absorptions. Similar changes were made in the second channel which monitored TO at 240 nm, and ollowed by TQ at 268 nm. The detector was operated at a sensitivity of 0.05 absorption unit full scale (AUFS).

For preparation of the mobile phase, methanol was glass distilled, filtered through a 0.45-μm α-metricel filter (Gelman Sciences, Ann Arbor, MI), and degassed under vacuum. Hexane and methylene chloride also were degassed under vacuum.

TABLE 1

Preparation of Stock Solutions and Working Solutions of α-T and TOP

Compounds	Dilutants	Stock solutions (μg/ml)	Working solutions (μg/ml)
α-Tocopherol	a	310	30.8 – 6.2
α-Tocopheryl quinone	a	450	45.0 – 0.9
Dihydroxy dimer	a	370	37.4 – 0.6
Dimer	a	580	58.0 – 2.4
Trimer	a	460	230.0 – 8.7
9-Methoxy-α-tocopherone	a	230	22.7 – 3.8

[a]Hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v).

Preparation of calibration curves of α-T and TOP. The stock solutions containing 310 μg α-T, 450 μg TQ, 370 μg DHD, 590 μg T and 230 μg TO/ml were diluted with the mobile phase to the desired concentrations for the preparation of standard curves (Table 1). A 60-μl of diluted standard solution was injected with a 100-μl blunt-needle Hamilton syringe (Rainin Instrument Co., Inc., Woburn, MA) onto the column. Peak heights and areas were recorded by a Spectra Physics 4270 computing integrator (Arlington, IL).

RESULTS AND DISCUSSION

Purification of TOP. Five TOP (TQ, D, DHD, T and TO) were synthesized and purified. After purification, each peak of the TOP was collected from a separate chromatographic run of a given sample on a reversed-phase C_{18} semipreparatory column and scanned from 400 nm to 200 nm. The absorbance maxima were found to be for α-T, TQ, DHD, D, T and TO at 292, 268, 292, 298, 294 and 240 nm, respectively, in the appropriate mobile phase. No changes were observed in absorbance maxima of α-T and the five TOP, when solvents were changed from the mobile phase of the C_{18} column to hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v), which is used for the normal-phase HPLC. The absorbance maxima were slightly different in the mobile phase for α-T and TOP when compared to the published data (5,24) because absorbances of α-T, DHD, D and T were measured in isooctane, and TO was measured in ethanol.

Separation of α-T and TOP by HPLC. A typical separation of α-T and the five TOP on a normal-phase column, using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) at a flow rate of 0.4 ml/min is demonstrated in Figure 1. Elution times for T, D, DHD and α-T recorded by the first channel were 5.3, 6.9, 7.5 and 14.0 min, respectively. Elution times for TO and TQ recorded by the second channel were eluted at 10.5 and 52.0 min, respectively. Widicus and Kirk (20) have reported the separation of TO and TQ by normal-phase HPLC, however, these compounds were closely eluted. The rest of the TOP, therefore, do not separate well in this system. In the present method, the elution time is about 40 min between TO and TQ, thus the TOP can separate very well.

Calibration curves. Calibration curves, prepared by injection of 50 μl working solutions, were obtained by measuring the peak height response (Figs. 2 and 3). The relationships of peak heights to the amounts injected under these conditions were linear in the tested range. The following linear regression equations were obtained: Y = 4438X + 125, α-T; Y = 8831X + 105, TO; Y = 33878X + 0.99, TQ; Y = 6312X − 251, DHD; Y = 4768X + 570, D; and Y = 500X + 20, T. Correlation coefficients were found to be greater than 0.999 for each compound. Correlations of peak areas to the amounts injected also were calculated but no differences were found in comparison

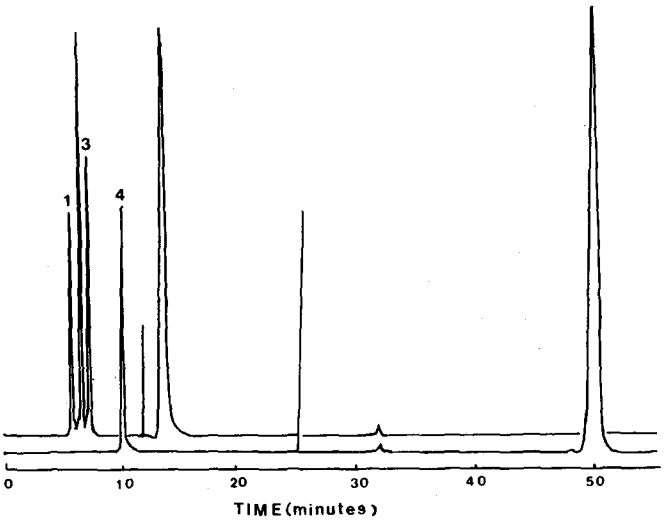

FIG. 1. Elution profile of α-tocopherol and its oxidation products on a 5-μm Ultrasphere-Si, 250 mm × 4.6 mm (i.d.). Peaks identification: trimer (1); dimer (2), dihydroxy dimer (3); 9-methoxy-α-tocopherone (4); α-tocopherol (5); α-tocopheryl quinone (6). Conditions: mobile phase, hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v); flow rate, 0.4 ml/min; detector with 295 nm for trimer, dimer and dihydroxy dimer, 292 nm for α-tocopherol, 240 nm for tocopherone and 268 nm for α-tocopheryl quinone; and attenuation at 0.05 AUFS.

FIG. 2. Calibration curves for high performance liquid chromatography of α-tocopherol (■—■), α-tocopheryl quinone (●—●) and 9-methoxy-α-tocopherone (O—O) on a 5-μm Ultrasphere-Si, 250 × 4.6 mm (i.d.). Data points represent the means of triplicate sample measurements; SEM was less than data point.

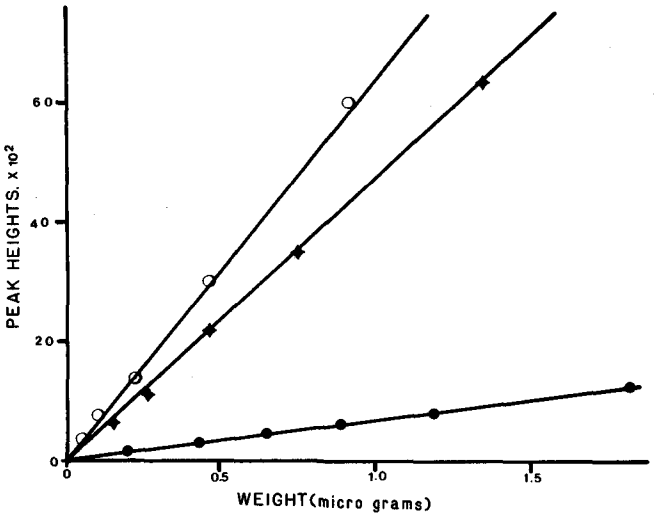

FIG. 3. Calibration curves for high-performance liquid chromatography of dihydroxy dimer (■—■), dimer (●—●), and trimer (○—○) on a 5-μm Ultrasphere-Si, 250 × 4.6 mm (i.d.). Data points represent the means of triplicate sample measurements; SEM was less than data point.

to peak heights. The lowest amounts detected by these newly developed normal-phase HPLC method were 0.01 μg for α-T, TQ and TO; 0.05 μg for DHD and D; and 0.1 μg for T/injection.

In summary, α-T, TQ, DHD, D, T and TO simultaneously were separated on a Ultrasphere-Si column using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v). This new normal-phase HPLC method has the following advantages compared to the previously published methods: the simultaneous separation of α-T and five TOP by a single chromatographic run, much higher sensitivity, and relatively longer retention time between TO and TQ ensuring good separation of TOP.

REFERENCES

1. Bieri, J.G., and Tolliver, T.J. (1981) *Lipids 16,* 777-779.
2. Csallany, A.S. (1971) *Intl. J. Vit. Nutr. Res. 41,* 376-384.
3. Csallany, A.S., Chiu, M., and Draper, H.H. (1970) *Lipids 5,* 63-70.
4. Csallany, A.S., and Draper, H.H. (1963) *Arch. Biochem. Biophys. 100,* 335-337.
5. Csallany, A.S., and Draper, H.H. (1963) *J. Biol. Chem. 238,* 2912-2915.
6. Strauch, B.S., Fales, H.M., Pittman, R.C., and Avigan, J. (1969) *J. Nutr. 97,* 194-202.
7. Draper, H.H., and Csallany, A.S. (1970) *The Fat Soluble Vitamins* (Deluca, H.F., and Suttie, J.W., eds.) pp. 347-353, University of Wisconsin Press, Madison, WI.
8. Buckley, J., and Connolly, J.F. (1980) *J. Food Protection 43,* 265-267.
9. Boguth, W., and Nienamm, H. (1971) *Biochim. Biophys. Acta 248,* 121-130.
10. Meijboom, P.W., and Jongentter, G.A. (1979) *J. Am. Chem. Soc. 56,* 33-35.
11. Osterlof, G., and Nyheim, P. (1980) *J. Chromatogr. 183,* 487-491.
12. Cillard, J., Cillard, P., and Cormier, M. (1980) *J. Am. Oil Chem. Soc. 57,* 252-255.
13. Chappel, J.E., Francis, T., and Clandinin, M.T. (1986) *Nutr. Res. 6,* 849-852.
14. Claude, F.B., George, P.R., and Cronenberger, L.A. (1984) *J. Assoc. Off. Anal. Chem. 67,* 627-632.
15. Zaspel, B.M., and Csallany, A.S. (1983) *Anal. Chem. 130,* 146-150.
16. Badcock, N.R., O'Reilly, D.A., and Pinnock, C.B. (1986) *J. Chromatogr. 382,* 290-296.
17. Bieri, J.G., Tolliver, T.J., and Catignani, G.L. (1979) *Am. J. Clin. Nutr. 32,* 2143-2149.
18. Cartignani, G.L., and Bieri, J.G. (1981) *Clin. Chem 29,* 708-712.
19. Stancher, B., and Zonta, F. (1983) *J. Chromatogr. 256,* 93-100.
20. Widicus, W.A., and Kirk, J.R. (1981) *J. Food Sci. 46,* 813-816.
21. Widicus, W.A., and Kirk, J.R. (1979) *J. Assoc. Off. Anal. Chem. 82,* 837-841.
22. Cillard, J., Gobaille, J., and Cillard, P. (1985) *J. Chromatogr. 347,* 434-437.
23. Eggitt, P.W.R., and Norris, F.W. (1956) *J. Sci. Food Agri. 7,* 493-511.
24. Boyer, P.D. (1951) *J. Am. Chem. Soc. 73,* 733-735.

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Microcomputer-based System for Data Acquisition and Analysis of Oxygen Uptake Studies

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The use of a Warburg Respirometer to study oxidation reactions is tedious and time-consuming. A microcomputer-based oxygen uptake device that is more sensitive than a Warburg Respirometer is described. The device consists of stainless steel or Teflon reaction vessels coupled to ultrasensitive pressure transducers. A high speed analog to digital convertor is used to poll the transducers. The direct memory access (DMA) controller of an IBM XT is used to poll the convertor and store the data in memory. Software used to program the DMA controller and IBM XT is described. The reproducibility and utility of the device is demonstrated with a brief study of lipid oxidation.
Lipids 23, 362-366 (1988).

The majority of the studies of oxidation of lipids in food and other biological systems entail monitoring the concentration of byproducts of the oxidation reaction. Compounds such as thiobarbituric reacting substances, conjugated dienes and volatiles classically are determined and correlated to the degree of oxidation (1). A more direct approach to evaluating the degree of oxidation in a food system is continuous determination of the rate of oxygen uptake. Assessment of oxygen uptake is faster and results in an accurate evaluation of oxidation in food systems. The Warburg Respirometer has been used in many classical studies of lipid oxidation in model food systems to monitor oxygen uptake during oxidation reactions (2–5) and is sensitive and accurately reflects the degree of oxidation in food systems. However, the major disadvantage of the Warburg Respirometer is that the change in pressure must be determined manually at appropriate intervals. This is tedious, time-consuming and results in collections of few data points over a long period of time. The purpose of this research was to develop, assemble and program a microcomputer–based device modeled after a Warburg Respirometer. The advantages of this device would be increased sensitivity, accuracy and resolution and be less labor-intensive.

MATERIALS AND METHODS

Reaction vessel. The reaction vessel in which the oxidation reactions take place was fabricated from 303 stainless steel. Figure 1 shows the basic design of the vessel. The volumes of the vessels were determined using standard Warburg Respirometer methods (6).

Pressure transducers. Low range pressure transducers (Setra Systems, Acton, MA, Model 239, ± 1 PSID) were fitted to the vessel to monitor pressure changes during oxidation.

Analog to digital convertor. An IBM XT compatible single board analog and digital input/output system (Data Translations, Marlboro, MA, Model DT2801/5714) was used to poll and convert the analog output from the transducers to a digital signal. A screw terminal and signal conditioning panel (Data Translations, Model DT707) was used to connect the transducers to the analog board. No signal conditioning is required with this A/D configuration.

Computer. An IBM XT, (IBM Inc., Boca Raton, FL, 10 Megabyte hard disk, 8087 math coprocessor, and color graphics adapter) and SixPacPlus (AST Research, Inc., Irvine, CA, 384K random access memory [RAM]) were used to acquire and process the data. An assembler (Microsoft, Corp., Redmond, WA, Macroassembler Version 4.0) and IBM Pascal compiler (IBM Inc., version 2.0) were used to compile the software.

Miscellaneous electronic components. A 60 Hz power line-driven clock was assembled to drive the A/D

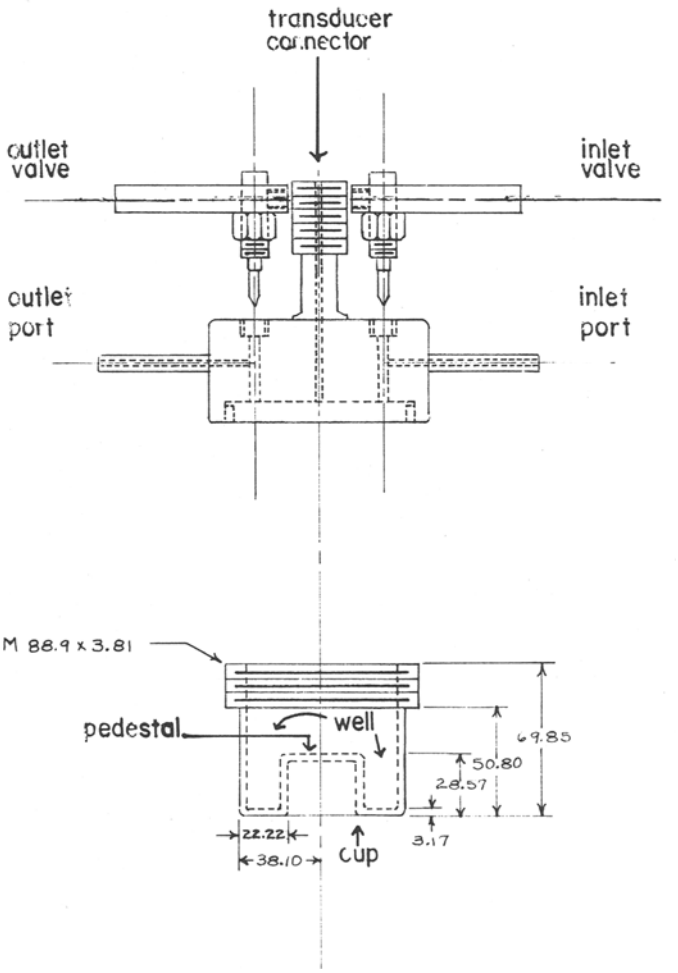

FIG. 1. Engineering details of reaction vessels.

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Abbreviations: CPU, central processing unit; DMA, direct memory access; RH, relative humidities.

convertor. A conventional regulated 24 volt DC power supply was used to drive the transducers. A standard pi filter was used to filter the input AC voltage for the clock, power supply and IBM XT.

Oxygen uptake experiment. The reproducibility of the device was evaluated by measuring the rate of oxygen uptake of a thin film of methyl linoleate equilibrated at two different relative humidities (RH). Three mg of methyl linoleate were spread on a 22 mm glass coverslip, placed in the reaction vessel and the vessel flushed with humidified oxygen. The inlet and outlet ports of the vessel were sealed, and oxygen consumption was monitored at 37 C for 48 hr. The data acquisition rate was set at one min.

RESULTS AND DISCUSSION

Reaction vessel. The reaction vessel used in this system was modeled after a glass Warburg flask. The stainless steel cylindrical vessel had a slightly larger volume than a Warburg Flask; however, the volume was kept as small as possible to increase the sensitivity of the apparatus (Fig. 1). The vessel contained a pedestal in the center of the cup to support a 22 mm cover slip above a reservoir that could be used to contain saturated salt solutions to control the relative humidity in the vessel. The sample was placed on a 22 mm circular glass cover slip and placed on the pedestal. The cover of the vessel has two Teflon-seated needle valves to facilitate flushing the system with air or other gases.

Glass vessels eliminate the possibility of corrosion by the salt solutions; however, the connection of metal transducers to glass vessels was not possible. Teflon reaction vessels also eliminate the possibility of corrosion and increase the utility of the system. A Teflon vessel without a pedestal would allow for magnetic stirring, and the system could be used for aqueous biological experiments. However, to assure there would be no change in volume with a change in pressure, the walls of a Teflon cup would have to be thick, and the poor heat transfer in Teflon results in a longer thermal equilibration time.

Pressure transducers. Of the transducers evaluated, only one met all of criteria set for the device. The Setra System Model 239 accurately senses a change in pressure as small as 1/100,000 of an atmosphere, 10 times more sensitive than the Warburg Respirometer (7). The transducer connects directly to the reaction vessels, increasing mobility, eliminating dead volume and the possibility of leaks at couplings and joints. Each transducer weighs eight oz and was supplied with an eight-foot cable, which gives greater mobility. The transducer is resistant to oxygen and other corrosive gases used in experiments, has an internal volume of 0.03 c in, has voltage regulation and thermal compensation circuitry allowing for operation at different temperatures.

The only disadvantage is that this transducer is a differential pressure sensor that necessitates the use of a thermobarometer. An absolute pressure sensor would be preferable and eliminate the need for a thermobarometer. An absolute pressure transducer that met the sensitivity requirements was not available, and therefore the differential transducer was selected.

Analog to digital convertor. The primary concern in selecting an analog to digital (A/D) convertor was matching the sensitivity of the convertor to the transducer. A 14-bit convertor discriminates one part in 120,000 of an atmosphere with this device. Other considerations were minimum eight channel differential inputs, complete software programmability, Direct Memory Access (DMA) data transfer capability, and programmable gain compatible with the pressure transducers.

A single board IBM XT compatible A/D convertor was chosen over a free–standing data logger because a single board unit was more cost effective. The IBM personal computer with a memory expansion unit has more than adequate memory to store the data. If the convertor has DMA capabilities, the microcomputer can be used to perform other tasks simultaneously with data acquisition and for considerably less cost than most data loggers.

Miscellaneous electronic components. The DT2801/5714 convertor has an internal programmable clock for periods of 5 μsec to 0.1638 sec. To allow for slower data acquisition, an external clock was assembled and used in place of the on-board clock. The period of external clock ranged from 0.2 to 6553 sec.

Figure 2 shows a schematic diagram of the complete oxygen uptake device. Up to eight transducers can be connected and monitored with the A/D convertor. The clock triggers the convertor to poll each transducer sequentially. The converted digital number is then output to a register and read by the microcomputer.

Software. The software necessary for data acquisition with a microcomputer is available widely in

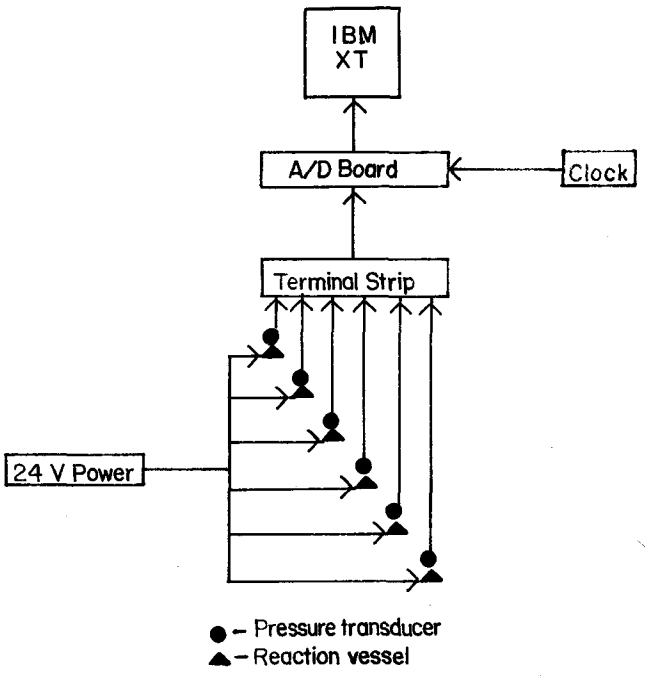

FIG. 2. Schematic drawing of complete oxygen uptake device.

varied price ranges. Because of the special capabilities on this system, special software was written. Several important factors were considered in writing the software for the device. The main goal in writing the software was not to dedicate the microcomputer solely to data acquisition. The microcomputer had to be multipurpose, available to perform other tasks such as data analysis simultaneous with data acquisition. The IBM PC and XT are capable of operation in the foreground/background mode.

The basic principle in running a microcomputer in the foreground/background mode is to use the 8237A-5 DMA controller in the IBM microcomputer for high-speed data transfers between the convertor and memory without processor intervention. Because the central processing unit (CPU) of the computer is not used during these operations, other processing can occur simultaneously. The DMA controller of the IBM microcomputers is user-accessible and can be programmed to acquire data from the convertor and transfer it to random access memory (RAM).

Programming the A/D convertor is done by sending commands to the appropriate register of the convertor. The registers and commands are specific for each convertor and generally explained in the documentation for specific convertors. Once the convertor is programmed and ready for the data acquisition, the DMA controller must be programmed and started before the convertor is started. Programming the DMA controller is similar to programming the convertor.

The DMA controller has four channels. One channel refreshes memory every 840 ns. Another channel is used to transfer data between memory and floppy disk drives, and if the microcomputer has a hard disk drive a third channel is used to transfer data from memory to hard disk. Therefore, an IBM PC has DMA channels 1 and 3 available for data acquisition while an XT only has channel 1 available. IBM (8) has a description of the 8237A-5 DMA controller.

The programming of the DMA controller was done with a Pascal procedure. First, channel 1 is initialized to read data from a peripheral device and write to memory in the autoinitialize mode. The autoinitialize mode is a continuous read from a device with no set number of bytes to be read. Next, the absolute address of data storage is sent to the controller. Several excellent books (9,10) or other computer texts give a detailed explanation of the segment and offset address system used by the IBM microcomputers. The data will be placed in memory starting at base address 0.

The controller is programmed to run in the autoinitialize mode. The controller will read continuously and write the data starting at base address 0, and automatically increment the base address for each byte read. The highest base address is 65535 or FFFFh, the maximum number of bytes the DMA controller can place in memory. After reading FFFFh bytes, the controller reinitializes and places data at memory base address 0. The result is that the controller will write over the existing data starting at address 0. This is a limitation of the computer and cannot be changed.

The remainder of the absolute memory address, segment or page is sent to the controller. The IBM PC/XT has 16 pages of 65536 bytes or 1,048,576 bytes of addressable memory. Some of these pages are reserved for special functions, such as video buffers and read only memory. Ten pages or ca. 640,000 bytes are available for storing data. Because the software must allow for the simultaneous data acquisition and use of the CPU for running other programs, memory must be reserved for both functions. Greater than 95% of computer software evaluated including the data analysis program used by this device used a maximum of four pages of memory. Four pages of memory were reserved for these programs, and six pages or approximately 400,000 bytes were reserved for data acquisition. The absolute requirement for memory during data acquisition is 64K bytes. The additional memory is used as storage of raw data prior to processing. There is more than adequate unused memory available under most conditions in a microcomputer, making a data logger unnecessary for data acquisition. Furthermore, the RAM of IBM microcomputers can be expanded to more than 8 mill. bytes by the addition of one or more Enhanced Expanded Memory System boards. Pages 4 through 9 were used for data acquisition. Before specifying the page address, the memory usage of commonly used programs should be evaluated. There are many programs available to evaluate memory usage. The authors can supply a program that performs this task.

The programmed DMA controller is started by sending the enable command. The final step is to start the programmed A/D convertor. The convertor is enabled to read continuously using the DMA controller and the external clock.

After starting both the convertor and controller, data acquisition is independent of the CPU, and the computer program used to program the controller and convertor can be terminated without affecting data acquisition. Other programs such as word processors, spreadsheets and data bases, if they do not use the memory locations specified for data acquisition, can be started and used simultaneously with data acquisition. The major disadvantage of running in the foreground/background mode is that data acquisition is free running and is not under the control of the CPU. It is not possible to modify the programming of either the controller or convertor without stopping all data acquisition.

It would be convenient to be able to add another channel for data acquisition without stopping all of the channels. This is not possible in the foreground/background mode. This problem was overcome by running all channels, whether a specific channel was being used to run an experiment or not. A new channel, one that currently is running but collecting useless data, is added by loading the reaction vessel of the channel and injecting a positive voltage spike from a 1.5 V battery to signify the start of the experiment or a negative spike to signify the end of the experiment. When the data is analyzed, data before a positive spike and after a negative spike is discarded and only experimental data is saved.

A Pascal program was used to retrieve and analyze data. The flowchart of the program is shown in Fig-

ure 3. The first part of the program polls the DMA controller to determine the number of bytes read. Second, the data is retrieved from RAM. The page number is entered by the user with the keyboard. The base address is always 0. Because the data is stored sequentially in RAM by the DMA controller, only the starting address is required and the base address is incremented by one after reading a byte. A data point consists of two bytes representing a 14-bit number. The data point is converted to a word, then integer, then real4 and finally converted to a voltage, pressure or concentration.

The data point is stored in the long heap using pointers. The data cannot be stored in variable arrays because the data segment of most interpreters and compilers is limited to 65536 bytes, which usually is exceeded in an experimental run. Therefore, the long heap was used temporarily to store the data until output to a disk. It is beyond the scope of this article to discuss the use of the long heap and pointers. There are several excellent books on the Pascal language (10,11). General data maintenance such as discarding nonsense bytes before or after a start/stop voltage spike are performed. The data can be analyzed further by linear or curvilinear least squares or other functions or stored on a disk in a format compatible with commonly used spread sheet or graphic programs. All functions can be performed simultaneously with data acquisition.

Although these programs are specific for the Data Translations A/D convertor and IBM XT, they can be modified to be used with other hardware. However, after modification they must be compiled with a IBM/Microsoft Pascal or other compatible compiler.[1]

Oxygen uptake experiment. To demonstrate the reproducibility of the device, the oxidation rates of thin films of methyl linoleate were determined. Figure 4 shows the change in oxygen pressure versus time for a typical uptake experiment. The initial nonlinearity in the uptake rate is due to thermal equilibration and is dependent on technique. Keeping the vessel at the

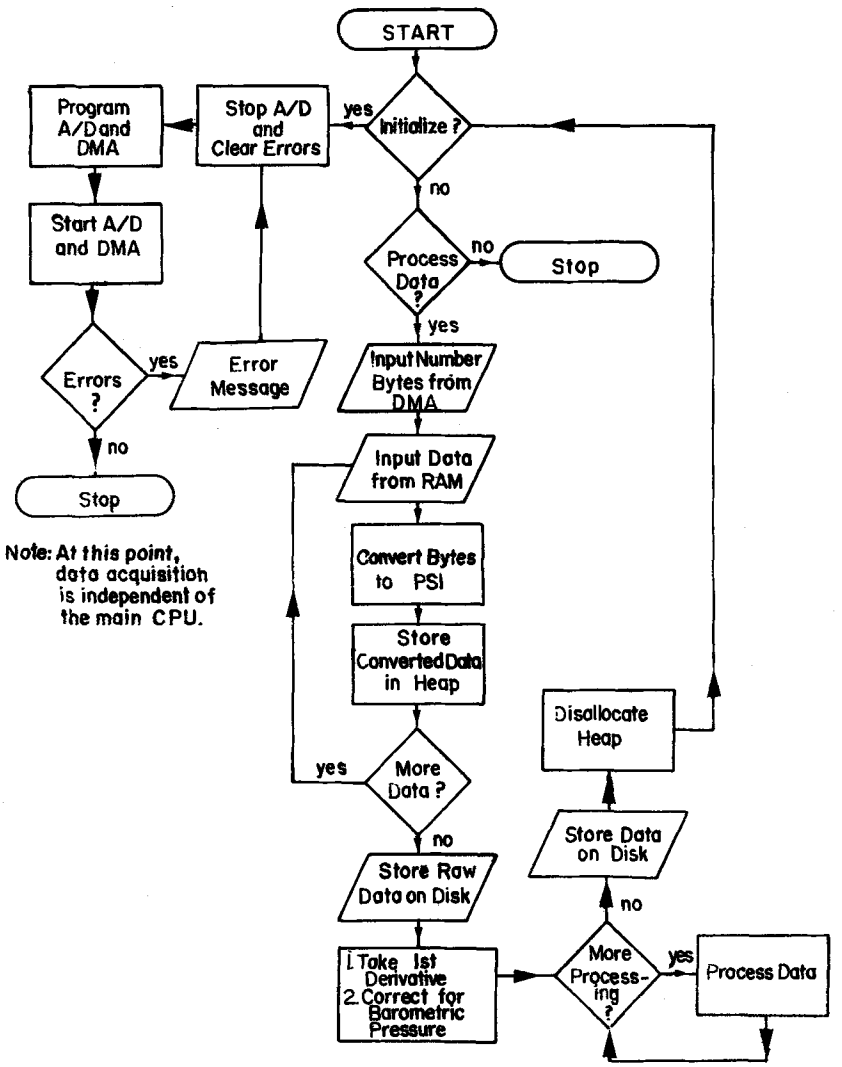

FIG. 3. Flow chart of data acquisition and analysis software.

[1]Copies of the data acquisition software and other utility programs can be obtained from the Department of Food Science and Human Nutrition, Washington State University, Pullman, WA 99164-6330, by submitting $5 to cover shipping and handling costs.

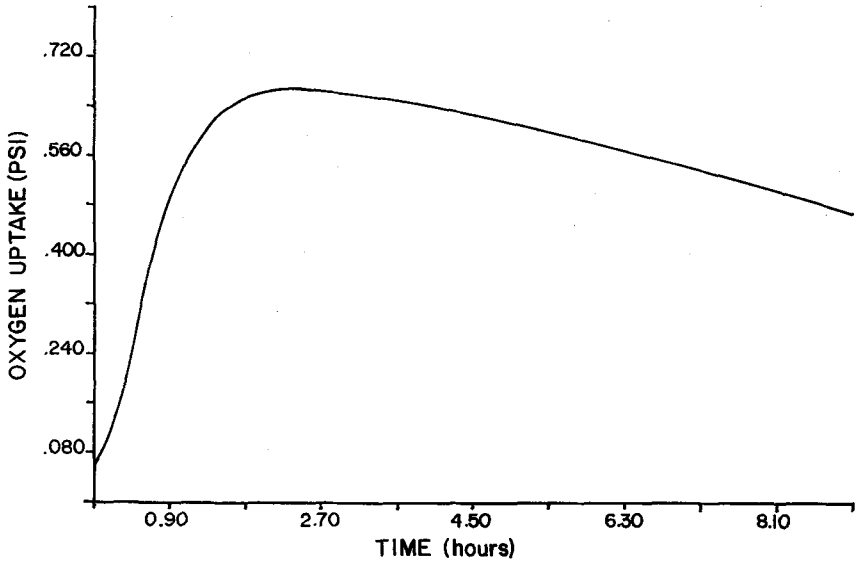

FIG. 4. Graph of change in oxygen pressure vs time of an oxidizing thin film of methyl linoleate.

experimental temperature during loading appreciably shortens the thermal equilibrium time. Thermal equilibrium and linearity in uptake rate usually is obtained within two hr. The use of differential pressure transducers necessitates the use of a thermobarometer. The transducers sense changes in barometric pressure, and the raw data must be corrected for these changes. A sealed empty vessel is used to obtain barometric pressure changes, which are subtracted from the raw data by the computer program used to retrieve data from memory.

The oxygen uptake rate was determined by linear least squares analysis of the linear portion of the curve. The oxygen uptake rates for thin films of methyl linoleate at two different relative humidities is shown in Table 1. The reproducibility of the runs is ca. 2–3%.

TABLE 1

Oxygen Uptake Rates by Thin Films of Methyl Linoleate at Different Relative Humidities

Relative humidity (%)	N	Oxygen uptake rate[a] (μmoles O_2/1 hr/mg Lipid)
0.0	4	.1041 ± 0.0032
50	11	.1727 ± 0.0030

[a]Plus or minus standard error of the mean.

In conclusion, the microcomputer-based oxygen uptake device has marked advantages over a Warburg Respirometer. The device is 10 times more sensitive and is more reproducible than the Warburg. The use of a microcomputer obviates the need for manual reading of the manometer and data entry into a computer. The computer is not dedicated to data acquisition because the computer is operated in the foreground/background mode. The result is that the computer can be used for data analysis, word processing or most other software simultaneous with data acquisition.

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REFERENCES

1. Gray, J.I. (1978) *J. Am. Oil Chem. Soc. 55*, 539–546.
2. Labuza, T.P., Maloney, J.F., and Karel, M. (1966) *J. Food Sci. 31*, 885–889.
3. Labuza, T.P., Silver, M., Cohn, M., Heidelbaigh, N.D., and Karel, M. (1971) *J. Am. Oil Chem. Soc. 48*, 527–531.
4. Labuza, T.P., Tsuyuki, H., and Karel, M. (1969) *J. Am. Oil Chem. Soc. 46*, 409–414.
5. Maloney, J.F., Labuza, T.P., Wallace, D.H., and Karel, M. (1966) *J. Food Sci. 31* 878–885.
6. Umbreit, W.W., Burris, R.H., and Stauffer, J.F. (1964) *Manometric Techniques*, 4th edn., Burgess Publishing Co., Minneapolis, MN, p. 46–51.
7. Setra Systems, Inc. (1985) Model 239 Technical Data Sheet, Setra Systems, Acton, MA.
8. IBM Corporation (1983) *Technical Reference for the IBM Personal Computer*, Revised Edition, Part Number 6025005, Ch. 1, pp. 1–3, 1–24, IBM Corp., Boca Raton, FL.
9. Lafore, R. (1984) *Assembly Language Primer for the IBM PC & XT*, pp. 231–272, Plume/Waite, New York, NY.
10. Pardee, M. (1984) *Pascal Primer for the IBM PC*, pp. 122–129, Plume/Waite, New York, NY.
11. IBM Corporation (1984) *Pascal Compiler Language Reference*, Version 2.0, Ch. 1, p. 10, IBM Corporation, Boca Raton, FL.

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COMMUNICATIONS

The Use of a Microwave Oven in the Chemical Transformation of Long Chain Fatty Acid Esters

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The use of a microwave oven as an energy source allowed transformation of fatty acid derivatives to be complete in a few minutes. Hydrolysis of triglycerides, esterification of free fatty acids, epoxidation of unsaturated fatty acid esters, cyclization of dioxostearates and oxounsaturated fatty acid esters into furanoid derivatives, conversion of epoxystearate to oxostearate derivatives, and substitution of a tosyl group by an azide all took about five min to complete and were in very high yields. The most sluggish reaction encountered was the acid catalyzed hydrolysis (ring opening) reaction of a furanoid ester to the corresponding 1,4-dioxo derivative, which required three 10-min periods of heating. The use of microwaves not only decreased the reaction time considerably but also allowed smaller volumes of solvent and lower quantities of reagents to be used as compared with the conventional methodologies reported.
Lipids 23, 367-369 (1988).

Microwaves are used extensively for telecommunication, industrial processes and biomedical applications (1,2). In research laboratories, microwave spectroscopy forms a powerful analytical technique for the determination of dipole moments in molecules and also in the study of internuclear distances and bond angles in certain molecules, in which changes in structural features hardly are observable by other methods (3). More recently, the use of microwave ovens in organic synthesis has been reported to reduce considerably the reaction time of an array of classical organic reactions (4,5). Here, we present results of a study of thermal reactions involving long chain fatty acid esters that normally would require many hours of heating or stirring at room temperature.

METHODS

A Sharp microwave oven, model R7370, 650 W, microwave frequency 2450 MHz, was used with a low setting. Reagents, substrates and solvents were placed in an autoclavable thick-walled, screw-capped pyrex test tube (20 ml). The charged test tube was embedded in vermiculite (a porous absorbant used as packing material for shipment of liquid chemicals) contained in a rectangular box (8 × 8 × 18 cm) designed with a beveled sliding lid and made of high-heat resistant Corian polymer (0.6 cm thickness, Dupont Inc., Wilmington, DE) provided by the Noland Co. The box was obtained through R. J. Giguere of the Department of Chemistry, Mercer University, Macon, GA. The box containing the reaction vessel was placed on the revolving dish of the microwave oven and irradiated for 1-10 min. After irradiation, the system was allowed to cool for five min inside the oven with the door opened. The plastic box then was removed to a fume hood, and the lid opened to allow further cooling for another five min. The product subsequently was isolated, purified and identified by methods described in the references in Table 1.

RESULTS AND DISCUSSION

Epoxidation of unsaturated fatty acid esters. When samples of methyl oleate were heated with *meta*-chloroperbenzoic acid in dichloromethane in a microwave oven for three and five min, respectively, gas liquid chromatography (GLC) analysis of the reaction products showed 93.6 and 98.8% conversion of the unsaturated substrate into the corresponding epoxy derivate. Thin layer chromatography (TLC) analysis of the reaaction product obtained from treatment of methyl linoleate for five min under similar conditions indicated a 100% conversion into the diepoxy derivative (methyl 9,10;12,13-diepoxystearate decomposed on GLC analysis). Methyl ricinoleate readily was converted in five min to methyl 12-hydroxy-*cis*-9,10-epoxystearate as shown by TLC analysis. The latter reaction furnished 82.5% of the product upon isolation.

Formation of C18 furanoid esters. When a mixture of methyl 12-oxo-*cis*-9-octadecenoate (obtained by oxidation of methyl ricinoleate) was heated for five min with mercuric acetate in acetic acid, 81.5% of the furanoid ester (methyl 9,12-epoxy-9,11-octadecadienoate) was obtained. A repeat reaction showed that a 97.3% conversion was attained after seven min of microwave heating. Two positional C18 furanoid fatty acid derivatives were obtained when methyl 9,10;12,13-diepoxystearate that was dissolved in a mixture of propyl iodide, sodium iodide and dimethylsulfoxide was subjected to microwave heating for five min. TLC analysis of the product showed complete transformation of the diepoxy substrate to the furanoid derivatives. Isolation of the product allowed 87.9% of the furanoid esters to be recovered. It appeared that not only did microwave treatment provide the thermal energy for the reaction, but since the reactions were carried out in sealed test tubes the pressure generated by the evaporating solvent also increased the reaction rates of these reactions.

Hydrolysis of triglycerides and esterification of free fatty acids. Hydrolysis of castor oil in the presence of KOH in ethanol and esterification of a sample of free fatty acid with 2% sulfuric acid in absolute methanol required only five min to complete. In both reactions, no double bond migration was observed, and no other by-products were produced.

Miscellaneous reactions involving fatty acid ester derivatives. The reaction of methyl *cis*-9,10-epoxystearate with a mixture of propyl iodide, sodium iodide and dimethylsulfoxide gave a mixture of methyl 9- and 10-

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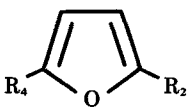
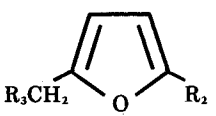
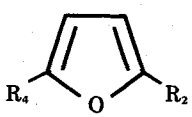
Abbreviations: GLC, gas liquid chromatography; TLC, thin layer chromatography.

TABLE 1

The Use of Microwaves in Thermal Reactions Involving Long Chain Fatty Acid Esters

Substrate (mmol)	Reagent (mmol)	Solvent	Reaction time (microwave)	Product	Yield %	Literature condition/yield	Ref.
R_1-CH=CH-R_2 (0.6)	*m*-Chloroperbenzoic acid (0.87)	CH_2Cl_2 (15 ml)	3 min 5 min	R_1-CH(O)CH-R_2	93.6 98.8	4 hr, 20 C, 100%	6
R_3-CH=CHCH$_2$CH=CH-R_2 (0.7)	*m*-Chloroperbenzoic acid (1.75)	CH_2Cl_2 (15 ml)	5 min	R_3-CH(O)CH-CH$_2$-CH(O)CH-R_2	100 (TLC)	12 hr, 20 C, 56%	7
R_4-CH(OH)-CH$_2$-CH=CH-R_2 (0.34)	*m*-Chloroperbenzoic acid (0.87)	CH_2Cl_2 (15 ml)	5 min	R_4-CH(OH)-CH(O)CH-R_2	100 (TLC) 82.5 (isol.)	12 hr, 20 C, 73%	7
R_4-C(=O)-CH$_2$-CH=*CH-R*$_2$ (0.36)	$Hg(OAc)_2$ (0.6)	AcOH (15 ml)	5 min 7 min		81.5 97.3	1 hr, reflux, 51%	7
R_3-CH(O)CH-CH$_2$-CH(O)CH-R_2 (0.32)	PrI,NaI, (2.4; 0.3)	DMSO (15 ml)	5 min	 R_3 CH_2R_2	87.9	5 hr, reflux, 43%	7
Castor oil (0.24)	KOH (3.8)	EtOH (10 ml)	5 min	free fatty acids	100	½ hr, reflux, 100%	8
FFA mixture (0.85)	H_2SO_4 (0.9)	MeOH (10 ml)	5 min	methyl ester of FA	100	½ hr, reflux, 100%	8
R_1-CH(O)CH-R_2 (0.32)	PrI,NaI (3.5; 0.5)	DMSO (15 ml)	5 min	$R_1CH_2CR_2$ + $R_1CCH_2R_2$	72.4	5 hr, reflux	9
R_4-CH(OTs)-CH$_2$-CH=CH-R_2 (0.47)	NaN_3 (1.2)	DMA (15 ml)	5 min	R_4-CH(N_3)-CH$_2$-CH=CH-R_2	85.7	4 hr, 90 C, 94%	10
 (0.41)	H_2SO_4 (10% aq.)	MeOH (10 ml)	10 min 20 min 30 min	$R_4CCH_2CH_2CR_2$	57.0 86.5 99.0	12 hr, reflux, 84%	11

R_1 = $CH_3(CH_2)_7$; R_2 = $(CH_2)_7COOCH_3$; R_3 = $CH_3(CH_2)_4$; R_4 = $CH_3(CH_2)_5$

oxostearates (72.4%) in five min. In a bimolecular nucleophilic substitution reaction of a tosyl group by azide, methyl 12-azido-*cis*-9-octadecenoate was obtained in 85.7% yield after five min from microwave treatment of methyl 12-tosyl-*cis*-9-octadecenoate with sodium azide in dimethylacetamide. The most sluggish reaction involving long chain fatty acid esters was found in the ring opening (acid-catalyzed hydrolysis) of a C18 furanoid fatty acid derivative with aqueous sulfuric acid in methanol. When the reaction mixture was heated in the microwave oven for 10 min, only 57% of methyl 9,12-dioxostearate was obtained. Upon reheating the cooled reaction mixture for another 10 min, the yield increased to 86%. Only when the reaction mixture was heated for another 10-min period was the conversion of the furanoid ester to the dioxo derivative complete. It was found necessary to heat the sealed test tube for periods not exceeding 10 min to prevent the reaction vessel from exploding, hence, the need to cool and reheat the reaction mixture after each 10-min microwave treatment.

The use of the microwave oven as a source of energy demonstrates the large savings in reaction time to the researcher. Beside obtaining high yields for these reactions, further savings were made by the use of much smaller volumes of solvents and lesser amounts of reagents per equivalent of substrate. While it appeared that most reactions seemed to benefit from this technique, we were unable to control the tosylation reaction of methyl ricinoleate with tosyl chloride. On heating of a mixture of tosyl chloride, methyl ricinoleate and dichloromethane, the analysis of the reaction product revealed an array of polar and nonpolar material. This reaction currently is being investigated further to identify the various products formed because tosylation of methyl ricinoleate was achieved readily without any heating when left at room temperature overnight.

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REFERENCES

1. Gandhi, O.P. (1981) *Microwave, Engineering and Applications*, pp. 4–9, Pergamon Press, New York, NY.
2. Gupta, K.C. (1979) *Microwaves*, pp. 2–4, Wiley Eastern Ltd., New Dehli, India.
3. Walker, S. (1976) in *Spectroscopy* (Straughan, B.P., and Walker, S., eds.) Vol. 2, pp. 76–136, Chapman and Hall, London.
4. Gedye, R., Smith, F., Westaway, K., Ali, H., Baldisera, L., Laberge, L., and Rousell, J. (1986) *Tetrahedron Lett. 27*, 279–282.
5. Giguere, R.J., Bray, T.L., Duncan, S.M., and Majetich, G. (1986) *Tetrahedron Lett. 27*, 4945–4948.
6. Gunstone, F.D., and Jacobsberg, F.R. (1972) *Chem. Phys. Lipids 9*, 26–34.
7. Lie Ken Jie, M.S.F., and Lam, C.H. (1977) *Chem. Phys. Lipids 20*, 1–12.
8. Lie Ken Jie, M.S.F., Lao, H.B., and Zheng, Y.F. (1988) *J. Am. Oil Chem. Soc. 65*, 597–600.
9. Bethell, D., Kenner, G.W., and Powers, P.J. (1968) *Chem. Commun.*, 227–229.
10. Lie Ken Jie, M.S.F., and Lao, H.B. (1987) *Chem. Phys. Lipids 45*, 65–74.
11. Lie Ken Jie, M.S.F., and Sinha, S. (1981) *Chem. Phys. Lipids 28*, 99–109.

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Malondialdehyde Excretion by Subjects Consuming Cod Liver Oil vs a Concentrate of n-3 Fatty Acids

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Urinary malondialdehyde (MDA), an indicator of lipid peroxidation in the diet and in the tissues, was determined in human adults consuming a supplement of n-3 fatty acids derived from a pharmaceutical grade of cod liver oil (CLO) without added antioxidants vs a concentrate of n-3 acids containing dodecyl gallate and vitamin E. MDA excretion increased immediately in the subjects consuming CLO but remained unchanged in those ingesting the concentrate for 50 days. The increase in the subjects taking CLO was attributable to MDA in the oil. The results indicate that consuming unstabilized fish oils as a source of n-3 fatty acids may entail exposure to potentially toxic products of lipid peroxidation.
Lipids 23, 370–371 (1988).

Fish oils and concentrates of n-3 fatty acids from fish oils have been widely promoted for the prevention of heart attacks. In as much as feeding fish oils is a classical method of inducing vitamin E deficiency in experimental animals, the question arises whether long-term ingestion of such oils may jeopardize the vitamin E status of humans. Long-term feeding of cod liver oil (CLO) to rats results in a significantly greater excretion of malondialdehyde (MDA) in the urine after a subsequent fast than is seen in rats fed corn oil, indicating an increase in peroxidation of fatty acids in vivo (1). This increase is inhibited partially but not entirely by feeding a high level of dietary antioxidants.

The possible presence in fish oils of peroxides and their degradation products including malondialdehyde, a mutagen (2) and reported carcinogen (3), is a further consideration in their long-term ingestion. Feeding a diet containing CLO to rats results in an immediate, marked increase in urinary MDA, reflecting a high level of this compound in the diet (4). MDA excretion in humans has been reported to increase following a meal of rancid foods (5).

In this study, the effect on MDA excretion by human adults of ingesting a commercial CLO product without added antioxidants vs a commercial fish oil concentrate containing a mixture of antioxidants was investigated.

METHODS

Experiment 1. Six adults consumed 30 ml of CLO (10 ml with each meal) for 14 days in conjunction with a free choice diet. The oil was a pharmaceutical product (Life Brand, Shoppers Drug Mart, Toronto, Ontario), labeled as a "natural source" containing no preservatives. On the basis of its reported content of eicosapentaenoic and docosahexaenoic acids (6% each) (6), this product provided about 1.5 g of each acid per day. After being opened, the bottles were kept at 4 C. Morning urine samples, one collected on the day before CLO ingestion began and the other on the last day of ingestion, were analyzed for MDA by the high performance liquid chromatography (HPLC) method of Draper et al. (1).

Experiment 2. Seven adults consumed a low-MDA diet (i.e. one devoid of meats and high-fat foods) and abstained from alcoholic beverages for three days, during which time consecutive morning urine samples were collected. For a further three days they consumed 30 ml of CLO, as in Experiment 1, in conjunction with a low-MDA diet, and three additional morning urine samples were collected for MDA analysis.

Experiment 3. Seven adults consumed 10 capsules per day of a concentrate of n-3 acids from fish oil (MaxEPA, Seven Seas Health Care Ltd., Kingston-upon-Hull, U.K.) for 50 days. The gelatin-coated capsules contained 100 ppm dodecyl gallate and provided 1.9 g eicosapentaenoic acid, 1.2 g docosahexaenoic acid and 10 IU of dl-α-tocopheryl acetate per day. Two consecutive 24-hr urine samples were obtained for MDA analysis immediately before supplementation began and on days 49 and 50. One day before and during each urine collection period, the subjects consumed a low-MDA diet.

RESULTS AND DISCUSSION

In Experiment 1, ingestion of CLO was associated with an increase in MDA excretion in all six subjects (Table 1). The mean increase of 37.5%, from 24.5 ± 3.5 μg to 34.7 ± 2.5 μg MDA (mean ± SEM), was significant ($P < 0.01$) using a two-tailed paired t-test.

In Experiment 2, CLO ingestion again was associated with an increase in MDA excretion in all subjects (Table 2). The mean increase of 54.3%, from 31.7 μg to 49.1 μg MDA/sample was highly significant ($P < 0.001$).

MDA excretion was unaffected by MaxEPA ingestion. Urinary MDA was 148 ± 15 μg/24 hr before

TABLE 1

Effect of Consuming a Pharmaceutical Grade of Cod Liver Oil[a] on MDA Excretion in the Urine (Experiment 1)

	MDA (μg)[b]	
Subject	−CLO	+CLO
1	29	36
2	12	31
3	35	38
4	31	44
5	18	26
6	22	33
$\bar{x}$	24.5	34.7[c]
SEM	3.5	2.5

[a]30 ml per day for 14 days.
[b]Morning void.
[c]$P < 0.01$.

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Abbreviations: CLO, cod liver oil; HPLC, high performance liquid chromatography; MDA, malondialdehyde.

TABLE 2

Effect of Consuming Cod Liver Oil[a] on Urinary MDA Excretion (Experiment 2)

Subject	MDA (μg)[b]	
	−CLO	+CLO
1	59	82
2	21	41
3	15	39
4	43	74
5	32	38
6	35	47
7	17	23
$\bar{x}$	31.7	49.1[c]
SEM	5.9	8.0

[a]30 ml per day for three days.
[b]Mean of three morning urine samples taken on consecutive days.
[c]$P < 0.001$.

TABLE 3

MDA Excretion by Subjects Consuming MaxEPA (μg/24 hr)

Subject	Diet	
	Free choice[a]	+MaxEPA[b]
1	130	160
2	216	201
3	153	118
4	154	113
5	123	109
6	113	133
$\bar{x}$	148	139[c]
SEM	15	15

[a]Mean of two 24-hr samples taken on consecutive days.
[b]After 50 days of supplementation.
[c]$P > 0.05$.

MaxEPA consumption and 139 ± 15 μg/24 hr after its consumption for 49–50 days (Table 3). This finding indicates that the fish oil concentrate was not a significant source of MDA in the diet or of MDA generated as a result of enrichment of the tissues with fatty acids of the n-3 series. It further indicates that the increase in MDA excretion seen during consumption of CLO was due to MDA present in the oil. Repeated extraction of a solution of CLO in hexane with 10% $NaHCO_3$ solution yielded sustained amounts of MDA, showing that the oil was undergoing continuous oxidative decomposition and further indicating that it was the source of the increase in MDA in the urine of the subjects consuming this product.

The proportion of ingested MDA excreted in the urine by humans is unknown but, judging from the results of studies on animals, it probably is small. Following stomach intubation with ^{14}C-MDA, rats excreted about 10% more ^{14}C in the urine than after intubation with ^{14}C-acetate (7). Sixty to 70% of the radioactivity was recovered in expired $^{14}CO_2$ within 12 hr.

Protracted consumption of n-3 fatty acids must be presumed to increase the vitamin E requirement. There is a need not only for an antioxidant capable of stabilizing these acids during storage but for a biologically active antioxidant capable of preventing their oxidation in vivo. Whether the normal diet provides enough vitamin E to meet the additional need for a biologically active antioxidant arising from consumption of n-3 fatty acids in unsupplemented fish oils is problematical. The results of this study indicate that the amounts of antioxidants present in MaxEPA are sufficient to suppress the oxidation of n-3 fatty acids in this product both in the capsules and in the tissues. They further indicate that ingestion of unstabilized fish oils entails a risk of exposure to potentially toxic products of n-3 fatty acid peroxidation. This is a particular consideration in jurisdictions, such as Canada, where the use of concentrates of n-3 fatty acids currently is prohibited.

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REFERENCES

1. Draper, H.H., Polensek, L., Hadley, M., and McGirr, L.G. (1984) *Lipids 19,* 836–843.
2. Basu, A.K., and Marnett, L.J. (1983) *Carcinogenesis (Lond.) 4,* 331–333.
3. Shamberger, R.J., Andreone, T.L., and Willis, C.E. (1974) *J. Natl. Cancer Inst. 53,* 1771–1773.
4. Piché, L.A. (1987) *Factors Affecting Malondialdehyde Levels in Rat and Human Urine and the Identification of N-ε-*(2-Propenal) Lysine as a Digestion Product of Food Proteins Exposed to Peroxidized Lipids, pp. 38–44, Ph.D. Thesis, University of Guelph, Ontario, Canada.
5. Jacobson, E.A., Newmark, H.L., Bird, R.P., and Bruce, W.R. (1983) *Nutr. Reports Int. 28,* 509–517.
6. Ahmed, A.A., and Holub, B.J. (1984) *Lipids 19,* 617–624.
7. Siu, G.M., and Draper, H.H. (1982) *Lipids 17,* 349–355.

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Differential Utilization of Long Chain Fatty Acids During Triacylglycerol Depletion. II. Rat Liver After Starvation

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Rats starved for 96 hr were shown to have a 94% reduction in liver triacylglycerol. Among the long chain fatty acids in liver triacylglycerol, only stearic acid and arachidonic acid were proportionally increased (2.5 and 6 times, respectively); palmitic and linoleic acids were unchanged, and palmitoleic and oleic acids were proportionally decreased. Stearic and arachidonic acids (mg%) were correlated positively within the triacylglycerol fraction, and both fatty acids varied inversely with total triacylglycerol (mg/g) in fed and starved rats. The utilization of long chain fatty acids from liver triacylglycerol during starvation resulted in selective retention of arachidonic acid and stearic acid and suggests that differential hydrolysis of liver triacylglycerol by hepatic lipase may occur or selective reacylation of these specific fatty acids may occur during starvation.
Lipids 23, 372–374 (1988).

The composition of arachidonic acid (20:4n-6, mg%) in rat liver triacylglycerol (TG) varies widely depending on the nutritional state of the animal (1). In liver and plasma, this variability has recently been shown to be a function of the total TG in liver or plasma (2,3); in starvation, low liver TG is associated with a higher proportion of 20:4n-6 in TG (4) and, in fatty liver syndromes, the reverse is seen (1,2). Although the proportional composition of 20:4n-6 in TG shows a significant inverse correlation with the total TG in individual animals within a species, the absolute composition of 20:4n-6 (μg/g tissue, μg/ml plasma) derived from TG is similar between individual animals. Hence, there is stability in the quantitative pool of 20:4n-6 in liver and plasma TG (2). With the exception of stearic acid (18:0), the apparent stability of the 20:4n-6 pool in TG is unique in comparison with 16- and 18-carbon fatty acids including linoleic acid (18:2n-6), which show no significant relation (positive or negative) with total TG or phospholipid in liver or plasma (2). This study has sought to determine whether, during starvation-induced depletion of liver TG, certain long chain fatty acids are utilized selectively while others, e.g. 20:4n-6, may be retained selectively in the TG.

EXPERIMENTAL

Twenty-four male Sprague-Dawley rats (200–250 g) were housed individually in polypropylene cages (Nalgene). They were fed a semi-synthetic diet of the recommended nutrient content: 20% casein, 50.5% sucrose, 15% cellulose, 10% corn oil (54% linoleic acid), 3.5% AIN-76 mineral mix and 1% AIN-76A vitamin mix (5). This diet was available for four wk along with distilled water given ad libitum. Food intake was determined once a week during this period (mean ± SD, 3.2 ± 0.7 g/100 g/day). Sixteen rats then were starved for 96 hr; eight rats continued to receive food ad libitum. The rats then were anesthetized with ether and the livers excised, blotted, weighed and frozen for lipid analysis.

The livers were homogenized in chloroform/methanol (2:1, v/v) containing 0.02% butylated hydroxytoluene as antioxidant. The TG component of the total lipid extract was separated by thin layer chromatography and transmethylated with boron trifluoride-methanol. The composition of the fatty acid methyl esters was determined by gas liquid chromatography (GLC) under the conditions specified (2). The quantitative content of TG in the liver was determined by GLC using triheptadecanoin as an internal standard that was added to the liver extract during homogenization.

Student's t-test was used for statistical evaluation of differences between groups.

RESULTS

The proportional composition of the major long chain fatty acids in the liver TG of fed and starved rats is shown in Figure 1. In comparison with fed rats, starved rats had a similar content (mg%) of palmitic acid (16:0) and

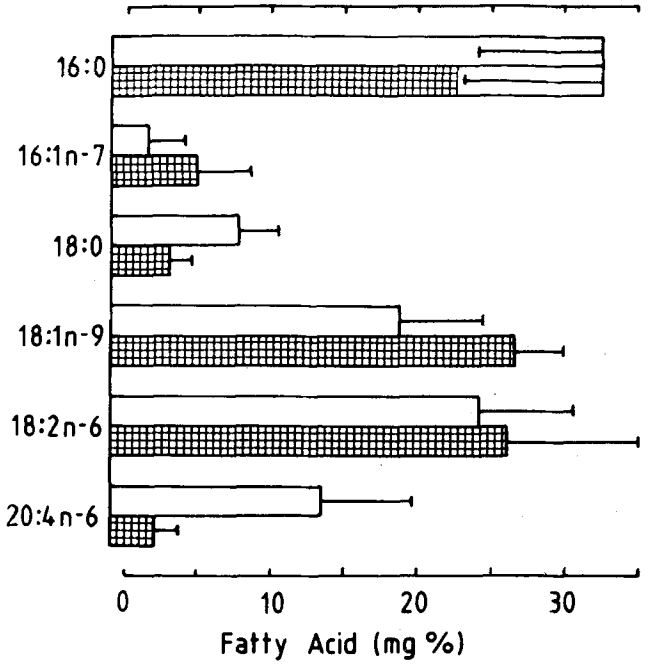

FIG. 1. Fatty acid composition (mean ± SD, mg%) of liver TG from 96-hr starved (open bar) or ad lib-fed (hatched bar) rats. In starved rats, palmitic acid (16:0) and linoleic acid (18:2n-6) were unchanged, palmitoleic acid (16:1n-7) and oleic acid (18:1n-9) were decreased ($p < 0.01$) and stearic acid (18:0) and arachidonic acid (20:4n-6) were increased ($p < 0.01$).

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Abbreviations: GLC, gas liquid chromatography; TG, triacylglycerol; 16:0, palmitic acid; 16:1n-7, palmitoleic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:2n-6, linoleic acid; 20:4n-6, arachidonic acid; 22:5n-3, docosapentaenoic acid; 22:6n-3, docosahexaenoic acid.

18:2n-6, a 29% reduction in oleic acid (18:1n-9, $p < 0.01$), a 67% reduction in palmitoleic acid (16:1n-7, $p < 0.01$), an increase in 18:0 of 157% ($p < 0.01$) and an increase in 20:4n-6 of 533% ($p < 0.01$). The total essential fatty acid content (18:2n-6 and 20:4n-6) of the liver TG was 25 mg% in the fed rats but was ca. 38 mg% in the starved rats (Fig. 1). Quantitation of the fatty acid data on a per g tissue basis (wet weight) showed that all the long chain fatty acids (16+ carbons) were decreased significantly in the liver TG of the starved compared with fed rats (Table 1). 16:0, 16:1n-7, 18:1n-9 and 18:2n-6 were present in the liver TG of the starved rats at <7% of their values in the fed rats, whereas 18:0 was present at 19% ($p < 0.01$) and 20:4n-6 at 34% ($p < 0.01$) of their respective values in the fed rats. The greatest difference between the starved and fed rats in the fatty acid composition (μg/g wet weight) of liver TG was in 16:1n-7 (−98%) and the smallest difference was in 20:4n-6 (−66%).

Only 20:4n-6 and 18:0 were proportionally higher in the liver TG of the starved compared with fed rats. To assess whether the relative retention of 20:4n-6 in the liver TG of the starved rats may have been related to that of 18:0, correlations were determined between these two fatty acids and total liver TG. Figure 2 shows that there was a significant inverse correlation between the total TG in liver and its content (mg%) of 18:0 and 20:4n-6. Hence, in fed rats (high liver TG) 20:4n-6 and 18:0 were both proportionally lower than in the starved rats (low liver TG). No other 16- or 18-carbon fatty acids were correlated significantly (positively or negatively) to the TG content of the livers of the starved or fed rats. In addition to being inversely correlated with total TG content, the proportional composition of 20:4n-6 and 18:0 was correlated positively (Fig. 3); in livers in which 20:4n-6 was low, 18:0 was also low and vice-versa. Interestingly, however, 20:4n-6 and 18:0 were not correlated in fed rats (high liver TG); they were only significantly correlated ($r = +0.70$, $p < 0.01$) in starved rats (low liver TG, Fig. 3).

TABLE 1

Absolute Composition of Long Chain Fatty Acids in Liver Triacylglycerol (TG) and Total TG of Fed or Starved Rats

Fatty acids	Ad lib fed[a]	96-Hr starved[b]	% Hydrolyzed
16:0	2000 ± 1607[c,d]	106 ± 70**	95
16:1n-7	327 ± 400	8 ± 7**	98
18:0	155 ± 101	30 ± 27*	81
18:1n-9	1496 ± 958	62 ± 54**	96
18:2n-6	1276 ± 946	79 ± 65**	94
20:4n-6	136 ± 117	46 ± 19**	66
TG	5.68 ± 3.49[e]	0.33 ± 0.24**	94

[a](n = 8).
[b](n = 16).
[c](μg/g).
[d](mean ± SD).
[e](mg/g wet weight).
*$p < 0.05$; **$p < 0.01$, Student's t-test.

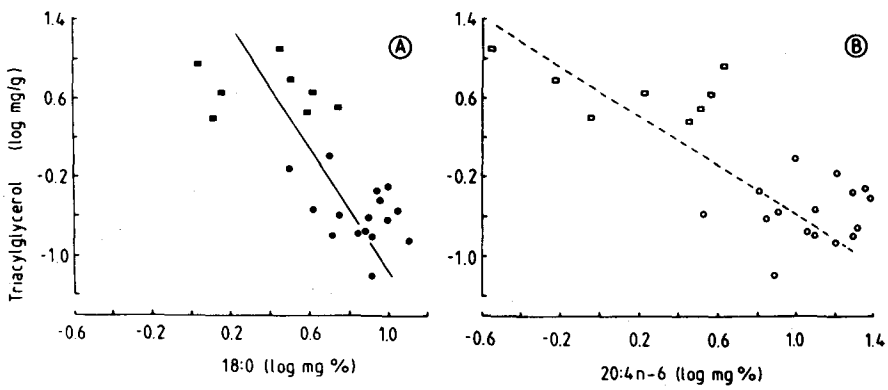

FIG. 2. Correlation regression analysis of the relation between liver TG (log mg/g) and its content of stearic acid (18:0, A; log mg%) and arachidonic acid (20:4n:6, B; log mg%) in ad lib-fed and 96-hr-starved rats. ■, stearic acid/fed; ●, stearic acid/starved; □, arachidonic acid/fed; ○, arachidonic acid/starved. Stearic acid − $r = -0.76$, $y = -0.34x + 0.66$, $p < 0.001$; Arachidonic acid − $r = -0.75$, $y = 0.96x + 0.58$, $p < 0.001$.

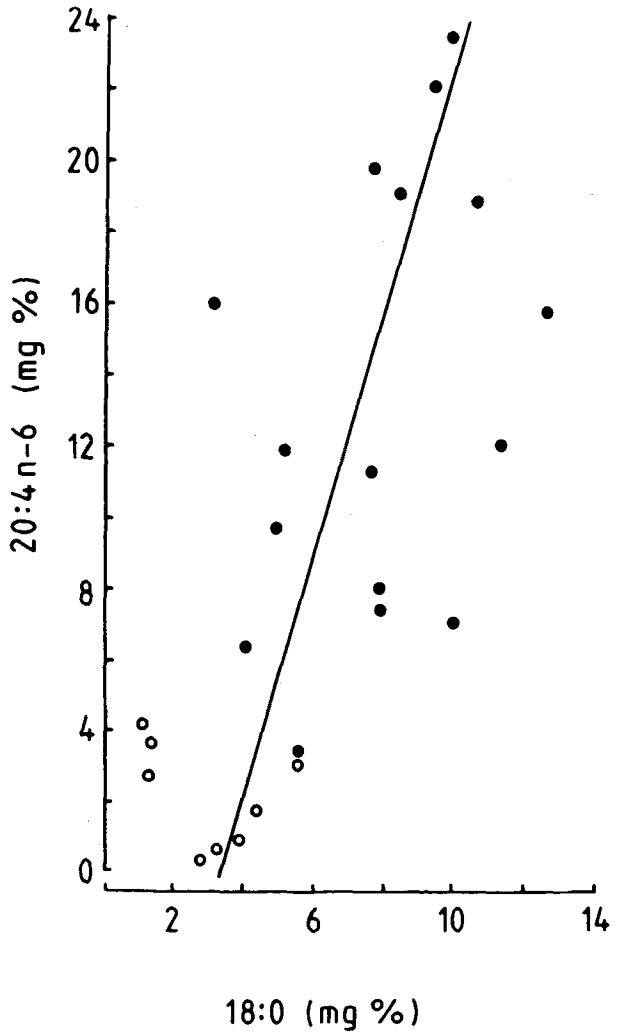

FIG. 3. Correlation regression analysis of the relation between the composition (mg%) of stearic acid and arachidonic acid in rat liver TG of 96-hr starved (●) and ad lib fed (○) rats: r = +0.65, y = 0.29x + 3.39, p < 0.001.

DISCUSSION

This study sought to establish whether the composition of long chain fatty acids in the total TG of the liver was altered differentially during starvation. The present data indicate that this is the case, particularly for 20:4n-6 and to a lesser extent for 18:0. As demonstrated (1-3), 20:4n-6 (mg%) also was found to be inversely correlated with total liver TG. Although the proportion of 20:4n-6 (mg%) was six times higher in starved vs fed rats, its absolute content was 66% less. 20:4n-6 is normally one of the smallest component fatty acids in liver TG of fed rats (1-2 mg%), but in the starved rats 20:4n-6 values of 20 mg% were not uncommon; 30 mg% have been observed previously (unpublished data). Hence, there was selective retention (on a proportional basis) of 20:4n-6 by rat liver TG during starvation.

These observations point to a marked differentiation between the proportional and absolute composition of 20:4n-6 in TG. It also is evident that long chain fatty acids are differentially hydrolyzed from liver TG or hydrolyzed and selectively reacylated during starvation with the following preference for hydrolysis: 16:1n-7 > 16:0 = 18:1n-9 = 18:2n-6 > 18:0 > 20:4n-6 (Table 1). The fact that net hydrolysis of 20:4n-6 was the least among these fatty acids would be expected in view of its unsaturation and chain length. The retention of 18:0 in the liver TG during starvation in amounts relatively greater than 18:1n-9 or 18:2n-6 was not expected. In view of the significant positive correlation between 20:4n-6 and 18:0 in the liver TG of the starved rats (Fig. 3), the proportional retention of 20:4n-6 and 18:0 may have been due to low specificity of the hepatic TG lipase for TG species containing 20:4n-6 and 18:0 or may have been due to net reacylation of these two fatty acids. These results are supported by a previous report showing that perfusion of intermediate density lipoproteins through the rat heart in vitro reduces their TG content by 60% (6). After perfusion, the percent composition of docosapentaenoic acid (22:5n-3) and docosahexaenoic acid (22:6n-3) in the TG of the lipoproteins was 2.2 times higher than before perfusion. It was concluded that differential hydrolysis of long chain fatty acids from lipoproteins by lipoprotein lipase occurs in the heart (6). These observations are analogous to the present TG data obtained from rat liver after starvation and suggest that TG depletion under various circumstances, e.g. from lipoproteins after perfusion through the heart (6), from endogenous stores in the perfused rat heart (7) or from rat liver during starvation (present data), results in selective retention not only of 20:4n-6 and possibly 22-carbon fatty acids but also of 18:0. The possibility that a significant proportion of 18:0 and 20:4n-6 are present in the same TG species in rat liver is currently being investigated.

ACKNOWLEDGMENTS

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REFERENCES

1. Cunnane, S.C. (1986) *Prog. Lipid Res. 26,* 91-92.
2. Cunnane, S.C., Huang, Y-S., and Manku, M.S. (1986) *Biochim. Biophys. Acta 876,* 183-186.
3. Cunnane, S.C., McAdoo, K.R., and Prohaska, J.R. (1986) *J. Nutr. 116,* 1248-1256.
4. Takeuchi, H., Ito, A., and Muramatsu, K. (1985) *Agric. Biol. Chem. 49,* 1853-1855.
5. American Institute of Nutrition (1977) *J. Nutr. 107,* 1340-1348.
6. Ridgway, N., and Dolphin, P.J. (1984) *Biochim. Biophys. Acta 796,* 64-71.
7. Cunnane, S.C. (1986) *Can. Fed. Biol. Soc. Proc. 29,* 127.

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Metabolism of 2(*RS*),3-Epiminosqualene to 24(*RS*),25-Epiminolanosterol by *Gibberella fujikuroi*

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The metabolic fate of 2*(RS)*,3-epiminosqualene (Isq.), a demonstrable inhibitor of the 2,3-oxidosqualene-lanosterol cyclase, has been studied in *Gibberella fujikuroi*. The fungus transforms Isq. to a tetracyclic product for which the 24*(RS)*,25-epiminolanosterol (EL) formulation was indicated by chromatographic and spectral data. The results are consistent with the pathway: Isq. → 2,3-imino-22, 23-oxidosqualene → EL.
Lipids 23, 375–376 (1988).

2,3-Epiminosqualene (Isq.) is a potent inhibitor of the enzymic cyclization of 2,3-oxidosqualene to sterols (1,2). This biochemical attribute has led to studies in which Isq. has been used to inhibit sterol biosynthesis in situ, thereby inhibiting fungal growth (3,4) and cell proliferation of cultured mammalian cells (5). Isq. also has been added to microsome preparations together with squalene analogues to assess the specificity of the enzymatically catalyzed epoxidation (6). The results described here show for the first time that Isq. is not a metabolically inert compound but may enter into the sterol pathway and undergo structural modifications analogous to squalene, viz. epoxidation and cyclization.

MATERIALS AND METHODS

Gibberella fujikuroi was incubated with 10 μg/ml Isq. for eight days as described (3). Authentic standards of Isq. and EL were prepared by the addition of iodine azide to the corresponding olefin (7). Mycelia undergoing growth adaptation were ground with sea sand and extracted overnight in a Soxhlet apparatus with refluxing acetone. The medium was extracted overnight with chloroform using a liquid-liquid apparatus. The mycelia and media extracts were monitored by thin layer chromatography (TLC) with the plates developed in solvent system I, benzene/ether, (9:1, v/v), and solvent system II, ethyl acetate/triethylamine, (99:1, v/v). The plates were sprayed with a 50% aq. H_2SO_4 solution and heated. R_f values for Isq. and EL in S.S.I and S.S.II were origin/origin and 0.42/0.25. Gas liquid chromatography (GLC) was performed with 3% SE-30 and 3% OV-17 packed columns operated isothermally at 245 C. Retention times were relative to cholesterol: RRT_c for Isq. and EL on the two columns was 0.80/0.70 and 2.63/3.21, respectively. Gas chromatography-mass spectrometry was performed using a 30 M DB-5 capillary column with the ion-source temperature for EI-MS maintained at 150 C. ^{1}HNMR was performed at 200 MHz as described (8). We noted that the authentic synthetically prepared iminoisopentenoids (7) produced a late shoulder on the parent GLC peak with varying intensity (from 0–40% of the parent peak). The formation of the shoulder is dependent on the hydroscopic state of the sample and has been observed also when

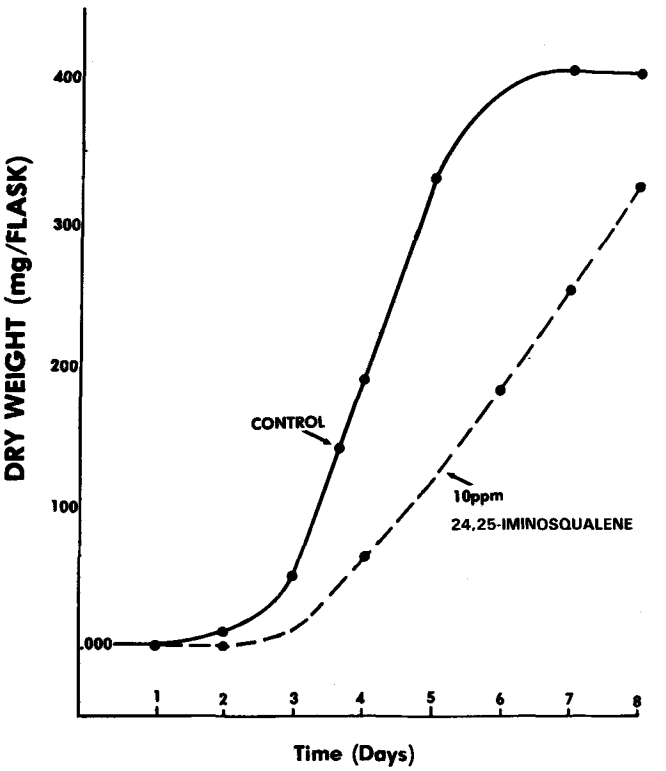

FIG. 1. Growth response of *Gibberella fujikuroi* with and without 10 μg/ml Isq. supplementation.

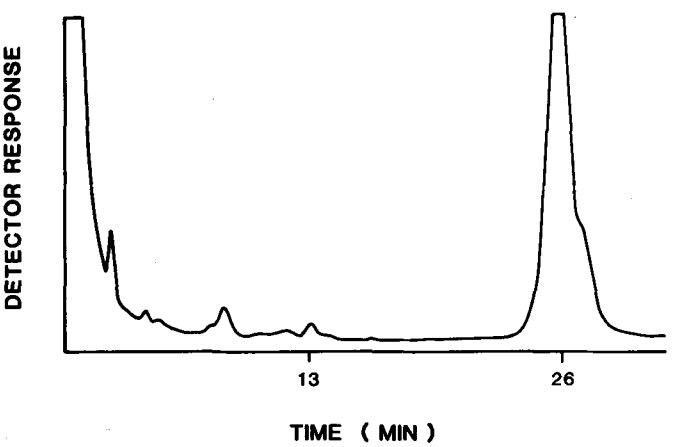

FIG. 2. GLC profile (3% SE-30 column operated at 245C) of the TLC band having R_f 0.25 in solvent system 2. Note the shoulder on peak at RRT_c-2.63 is a breakdown product. See text for details.

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Abbreviations: AL, 25-aminolanosterol; EL, 25-epiminolanosterol; Isq., 2,3-Epiminosqualene; GLC, gas liquid chromatography; TLC, thin layer chromatography.

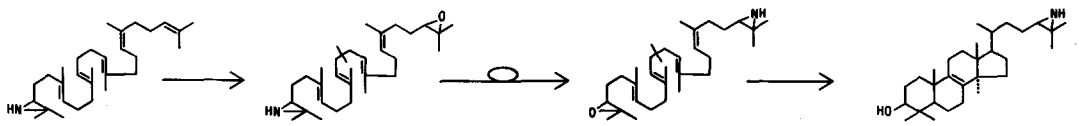

FIG. 3. Proposed 2,3-iminosqualene pathway in *Gibberella fujikuroi*.

the injector port accumulates bound water and needs to be cleaned.

RESULTS AND DISCUSSION

In recent studies using *G. fujikuroi*, we demonstrated that the fungus produced ergosterol and other 24β-methylsterol end products (3,8). Isq. added to the cultures at 1 μg/ml inhibited growth somewhat and induced the fungal accumulation of squaleneoxide and the depression of [2-^{14}C]acetate incorporation into sterol end products (3). When the level of Isq. was increased to 10 μg/ml, mycelial growth ceased. However, after a few days of chronic treatment with Isq. the mycelia recovered and grew in a manner analogous to control cultures. We now have repeated the study to determine whether a developmentally delayed biochemical detoxifying mechanism operates to modify Isq. to form a noninhibitory end product.

Isq. was incubated at 10 μg/ml for eight days with *G. fujikuroi* as shown in Figure 1. The mycelia initially were inhibited, then growth resumed. The cultures at harvest apparently had recovered to the inhibitor at an earlier time than we had shown in a previous study (3). Media extracts that were monitored for Isq. at harvest failed to possess significant levels of the dietary Isq. However, mycelial extracts contained in addition to Isq. a major unidentified compound that was visible in the second TLC system and that was sensitive to the separation of N-isopentenoids i.e., at R_f 0.25. This material eluted in GLC with authentic EL (Fig. 2) and exhibited a characteristic fragmentation pattern for an aziridine steroid: M^+, 441(62%), 426(100%), 409(6%), 400(9%), 166(27%), 99(62%), 98(57%), 84(78%), 70(45%), 58(28%); ^{1}HNMR δ C-18(0.69s), C-19(1.00s), C-21(0.91d), C-26(1.25s), C-27(1.16s), C-30(0.98s), C-31(0.81s), C-32(0.88s) ppm. The amount of EL per culture flask was quantified at 10 μg/80 mg dry wt mycelium. At physiological pH, the protonated aziridine is a reactive species capable of undergoing a ring-opening reaction with a nucleophile. Through a biomimetic approach, we have shown that 24,25-epiminolanosterol is converted to 25-aminolanosterol using hydrogen and Raney nickle (9). Thus, the continued transformation of the aziridinium salt to 25-aminolanosterol (AL) is possible; preliminary evidence in fact has been presented for the EL to AL conversion by *G. fujikuroi* (10). Studies in progress should resolve whether deamination of the 25-amino grouping occurs; this would effectively produce a nontoxic metabolite, viz. a Δ^{24}-steroid, which then could reenter into the sterol C-24 alkylation pathway.

The biosynthetic scheme presented in Figure 3 for the metabolic conversion of Isq. to EL is consistent with the mechanistic considerations implicit in the studies cited (6,11-13).

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REFERENCES

1. Corey, E.J., de Montellano, P.R.O., Lin, K., and Dean, P.D.G. (1967) *J. Am. Chem. Soc. 89*, 2797-2798.
2. Duriatti, A., Bouvier-Nave, P., Benveniste, P., Schuber, F., Delprino, L., Balliano, G., and Cattel, L. (1985) *Biochem. Pharmacol. 34*, 2765-2777.
3. Nes, W.D., and Heupel, R.C. (1986) *Arch. Biochem. Biophys. 244*, 211-217.
4. Pinto, W.J., and Nes, W.R. (1983) *J. Biol. Chem. 258*, 4472-4476.
5. Popjak, G., Meenan, A., and Nes, W.D. (1987) *Proc. R. Soc. Lond. B 232*, 273-287.
6. Van Tamelen, E.E., and Heys, J.R. (1975) *J. Am. Chem. Soc. 97*, 1252-1253.
7. Parish, E.J., and Nes, W.D. (1988) *Synth. Commun. 18*, 221-226.
8. Nes, W.D., Heupel, R.C., and Le, P.H. (1985) *J. Chem. Soc. Chem. Commun.*, 1431-1433.
9. Parish, E.J., Hanners, P.K., and Nes, W.D. (1987) in *The Metabolism, Structure and Function of Plant Lipids* (Stumpf, P.K., Mudd, J.B., and Nes, W.D., eds.) Plenum Press, New York, NY, pp. 103-105.
10. Nes, W.D., Hanners, P.K., and Parish E.J. (1986) *Biochem. Biophys. Res. Commun. 139*, 410-416.
11. Shishibori, T., Fukui, T. and Suga, T. (1973) *Chem. Lett. (Japan)*, 1289-1292.
12. Corey, E.J., and Gross, S.K. (1967) *J. Am. Chem. Soc. 89*, 4561-4562.
13. Cattel, L., Ceruti, M., Viola, F., Delprino, L., Balliano, G., Duriatti, A., and Bouvier-Nave, P. (1986) *Lipids 21*, 31-38.

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Fatty Acids of the Milk and Food of the Platypus (*Ornithorhynchus anatinus*)

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Platypus milk fat contains 98.5% triglyceride. Polyunsaturates (C18:2–C22:5) account for 29% of the triglyceride fatty acids in the milk fat and 32% of the total fatty acids in the lipid of the food of the platypus. Linoleate and arachidonate are the major ω6 polyunsaturates of both food and milk lipids. However, while the ω3 polyunsaturates linolenate and eicosapentaenoate are present in both food and milk, docosapentaenoate is present in meaningful amounts in milk only. It is suggested that with the exception of 22:5ω3, the polyunsaturates in platypus milk originate in the diet.

Lipids 23, 377–379 (1988).

Platypuses *(Ornithorhynchus anatinus)* living in the waters of South Eastern Australia breed in spring and lay their eggs early in September. This takes place in a burrow in which the progeny live solely on milk for at least 3.5 months until they emerge in late January or in February. The milk triglyceride contains relatively large amounts of long-chain polyunsaturates (1–4). In contrast to this, the milk triglyceride of the echidna, an egg-laying mammal related to the platypus, living on its natural diet of ants and termites contains linoleic and linolenic but, apart from a trace of arachidonic acid, no long-chain polyunsaturates of 20 or 22 carbons (1,4). Total fatty acids of the lipids of a species of ant and of a termite eaten by echidnas have been determined, and the C20–C22 long-chain polyunsaturates are absent (4). Since the fatty acid composition of echidna milk fat can be changed by altering the composition of the fatty acid intake (1,2,4), it is possible that platypus milk fat differs from that of echidnas due to a difference in fatty acid intake. To test this notion, the fatty acid composition of the lipid in the food of the platypus was determined. Since the classes of lipid in platypus milk, other than triglyceride measured by yield from Florisil columns, have never been defined, these also were determined.

MATERIALS AND METHODS

Eleven lactating platypuses were captured in the Shoalhaven River N.S.W. by the method of Grant and Carrick (5) during a three-day period in mid-December 1984 and injected intramuscularly with 0.2 ml (2 I.U.) of oxytocin (Sandoz). Milk then was squeezed out of the mammary glands and taken up in Pasteur pipettes. The cream was separated from each sample by centrifugation at 1,000 × g for 20 min at 2–4 C. It then was removed and stored under nitrogen at −20 C.

The diet of the platypus in the Shoalhaven consists mainly of aquatic insect larvae (Trichoptera, Diptera, Coleoptera, Ephemeroptera, Odonata) shrimps and bivalve molluscs (6). Samples of available items were taken in the winter (3/7/84), summer (8/12/85) and autumn (15/3/86) by means of a Surber Sampler and a plankton net in the riffle areas and pools in which the lactating platypuses were trapped. Upon removal from the nets, the pooled samples of organisms were frozen and stored at −10 C.

The milk fat was extracted by the method of Gibson and Kneebone (7) and the classes of lipid isolated by thin layer chromatography. The fatty acids of the lipids were transesterified in 1% H_2SO_4 in methanol (v/v) by heating to 70 C for three hr (8); the resultant methyl esters were analyzed by gas chromatography (7).

The lipid of the pooled samples of food items was extracted by the method of Bligh and Dyer (9).

RESULTS AND DISCUSSION

Thin layer chromatography showed that 98.5% of the milk lipid was in the form of triglyceride; the remainder was made up of phospholipid (1%) and free fatty acids. Very small amounts of cholesteryl esters were detectable in five of the samples only. Data in Table 1 show that unsaturates predominate in all four classes of lipid, accounting for over two-thirds of the fatty acids present in the milk fat. Of these 29% of the total present in the triglyceride are polyunsaturates (C18:2–C22:5), in agreement with the data of Griffiths et al. (1) and Grant et al. (3), which give 27% and 32%, respectively. From Table 1, it also can be seen that the concentration of arachidonic acid (20:4ω6) in the phospholipid is higher than that in the triglyceride; however, 98% of the total arachidonic acid is in the latter fraction. The greater part of the arachidonic acid present in human milk fat is also present in the triglyceride (10,11).

The fatty acid composition of the lipid in the pooled samples of platypus food is shown in Table 2. From this, it is clear that about 60% of the fatty acids are unsaturates; polyunsaturates account for about 32% of the total fatty acids. Because it is known that dietary intake of polyunsaturates by lactating women leads to the appearance of those acids in their milk (8,12–14), it would be fair to conclude that the presence of polyunsaturates in platypus milk is due to ingestion of those acids present in their food. Likewise, it affords an explanation of the absence of long-chain polyunsaturates from the milks of echidnas because their food lipid apparently lacks C20-C22 polyunsaturates (4). However, while 18:2ω6, 20:4ω6, 18:3ω3 and 20:5ω3 were present in high amounts in both food and milk lipids, 22:5ω3 was present in large amounts in milk only. Because 22:5ω3 can be converted readily from 20:5ω3, it is likely that some chain lengthening of fatty acids has occurred, most likely in the liver.

Small amounts of 22:6ω3 were found in platypus food lipids and in their milk lipid. However, Griffiths et al. (1) reported the presence of 0.6–1.1% of 22:6ω3 in milk triglyceride of platypuses living in habitats different from

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TABLE 1

Fatty Acid Composition (Weight %) of the Classes of Lipid in Platypus Milk

Fatty acid	Triglyceride	Phospholipid	Free fatty acids	Cholesterol esters[a]
Saturates				
14:0	1.6 ± 0.4	1.6 ± 0.6	1.2 ± 0.3	1.9 ± 0.4
15:0	0.7 ± 0.7	0.6 ± 0.7	0.4 ± 0.3	0.5 ± 0.1
Iso 16:0	0.2 ± 0.2	1.5 ± 0.3	0.3 ± 0.2	0.6 ± 0.4
16:0	19.8 ± 0.8	23.6 ± 2.5	9.6 ± 9.4	19.0 ± 1.7
17:0	1.8 ± 0.3	1.4 ± 0.3	1.9 ± 0.4	1.5 ± 0.2
Iso 18:0	0.3 ± 0.4	2.1 ± 0.3	1.7 ± 1.2	0.1 ± 0.2
18:0	3.9 ± 0.5	9.1 ± 1.5	2.5 ± 0.5	4.0 ± 0.5
20:0	1.3 ± 0.2	1.6 ± 0.2	1.8 ± 0.5	1.1 ± 0.2
22:0	0.5 ± 0.1	2.5 ± 0.6	0.7 ± 0.1	0.5 ± 0.1
24:0	0.2 ± 0.1	1.1 ± 0.4	0.2 ± 0.1	0.4 ± 0.2
Monounsaturates				
14:1	0.7 ± 0.1	0.5 ± 0.2	0.8 ± 0.4	0.6 ± 0.1
16:1	13.9 ± 1.4	11.2 ± 1.5	15.7 ± 1.9	12.8 ± 0.7
17:1	1.5 ± 0.9	0.5 ± 0.4	0.6 ± 0.4	1.2 ± 0.2
18:1	22.7 ± 2.6	15.0 ± 1.4	19.8 ± 3.9	25.6 ± 2.2
20:1	2.2 ± 0.4	0.9 ± 0.3	1.2 ± 0.5	2.3 ± 0.3
24:1	tr	tr	0.2 ± 0.1	0.4 ± 0.1
Polyunsaturates				
ω6 series				
18:2	5.4 ± 0.8	4.5 ± 0.6	7.9 ± 1.4	7.9 ± 0.6
18:3	0.6 ± 0.1	0.3 ± 0.1	0.7 ± 0.2	0.9 ± 0.1
20:2	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.0
20:3	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0
20:4	2.4 ± 0.3	5.5 ± 1.0	3.3 ± 0.4	2.1 ± 0.3
22:4	0.4 ± 0.1	0.2 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
ω3 series				
18:3	7.6 ± 1.9	4.6 ± 2.1	15.2 ± 4.0	6.4 ± 2.2
20:5	4.5 ± 0.6	4.2 ± 0.8	4.6 ± 1.1	4.3 ± 0.8
22:5	4.2 ± 0.5	5.6 ± 1.6	5.8 ± 1.0	4.1 ± 0.8
22:6	tr	0.8 ± 0.5	0.2 ± 0.4	ND
Unidentified	2.8 ± 0.6	ND	1.9 ± 1.1	ND

n, 11; Mean ± S.D.

[a]n, 5.

tr, trace; ND, not detected.

TABLE 2

Fatty Acid Composition (Weight %) of Platypus Food Lipid Sampled in Winter, Summer and Autumn

	Sample		
Fatty acids	Winter 3/7/84	Summer 18/12/85	Autumn 15/3/86
Saturates			
14:0	1.9	2.2	1.2
15:0	0.4	0.2	0.3
Iso 16:0	0.3	0.5	0.2
16:0	19.9	27.4	25.4
17:0	2.3	2.0	2.1
Iso 18:0	1.3	1.1	1.3
18:0	6.5	4.6	5.5
20:0	1.6	1.8	1.6
22:0	0.4	0.3	0.6
24:0	0.1	0.1	0.1
Monounsaturates			
14:1	0.7	0.7	0.4
16:1	10.2	14.5	9.9
17:1	0.6	0.3	0.3
18:1	21.3	17.5	17.2
Polyunsaturates			
ω6 series			
18:2	7.0	4.8	7.5
18:3	0.5	0.4	0.9
20:2	0.1	0.1	0.1
20:3	0.1	0.1	0.1
20:4	2.2	1.0	2.7
ω3 series			
18:3	10.4	13.5	13.7
20:5	11.8	6.4	8.5
22:5	tr	0.1	tr
22:6	0.1	0.3	0.2

tr, trace; n, 1.

the Shoalhaven River. The discrepancy may be due to differences in the levels of 22:6ω3 in the food items selected by the animals. In addition, we report here less 12:0 and 14:0 in the triglyceride fraction than reported in an earlier study (15). While this may be due to seasonal or biological differences, we are satisfied that the methylation procedure used in this study does not cause selective losses of C12 and C14 fatty acids.

The origin of the polyunsaturates in the food of the platypus is unknown but the ultimate foodstuffs of aquatic molluscs and insect larvae are detritus and algae (16). It is known that benthic marine algae contain long-chain polyunsaturated fatty acids (17), so it is not unlikely that fresh water algae will prove to be the primary source of C20-C22 polyunsaturates in platypus milk.

REFERENCES

1. Griffiths, M., Elliot, M.A., Leckie, R.M.C., and Schoefl, G.I. (1973) *J. Zool. Lond. 169,* 255-279.
2. Griffiths, M. (1978) in *The Biology of the Monotremes,* pp. 280-299, Academic Press, New York, NY.
3. Grant, T.R., Griffiths, M., and Leckie, R.M.C. (1983) *Aust. J. Zool. 31,* 881-889.
4. Griffiths, M., Green, B., Leckie, R.M.C., Messer, M., and Newgrain, K.W. (1984) *Aust. J. Biol. Sci. 37,* 323-329.
5. Grant, T.R., and Carrick, F.N. (1974) *Aust. Zool. 18,* 133-135.
6. Faragher, R.A., Grant, T.R., and Carrick, F.N. (1979) *Aust. J. Ecol. 4,* 171-179.
7. Gibson, R.A., and Kneebone, G.M. (1981) *Am. J. Clin. Nutr. 34,* 252-257.
8. Kneebone, G.M., Kneebone, R., and Gibson, R.A. (1985) *Am. J. Clin. Nutr. 41,* 765-769.
9. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
10. Crawford, M.A., Sinclair, A.J., Msuya, P.M., and Munhambo, A. (1973) in *Dietary Lipids and Postnatal Development,* pp. 41-56, Raven Press, New York.
11. Gibson, R.A., and Kneebone, G.M. (1984) *Lipids 19,* 469-471.
12. Insull, W., Hirsch, J., James, T., and Ahrens, E.H. (1959) *J. Clin. Invest. 38,* 443-450.
13. Vuori, E., Kiuru, K., Makinen, S.M., Vayrynen, P., Kara, R., and Kuitunen, P. (1982) *Acta Paediatr. Scand. 71,* 959-963.
14. Harris, W.S., Conner, W.E., and Lindsey, S. (1984) *Am. J. Clin. Nutr. 40,* 780-785.
15. Parodi, P.W., and Griffiths, M. (1983) *Lipids 18,* 845-847.
16. Cole, G.A. (1983) *Textbook of Limnology,* 3rd edn., pp. 164-167, C.V. Mosby Co., St. Louis, MO.
17. Johns, R.B., Nichols, P.D., and Perry, G.J. (1979) *Phytochemistry 18,* 799-802.

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Fatty Acid Composition of Macrophage Phospholipids in Mice Fed Fish or Borage Oil

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The polyunsaturated fatty acid (PUFA) composition of murine peritoneal macrophage phospholipids was dramatically altered in vivo following the four-wk feeding of specific dietary oils. Fish oil (containing 20:5n-3 and 22:6n-3) feeding significantly increased macrophage 20:5n-3, 22:5n-3, and 22:6n-3 ($P < 0.05$), while borage oil (containing 18:2n-6 and 18:3n-6) increased ($P < 0.05$) the macrophage 20:3n-6/20:4n-6 ratio, relative to safflower oil (containing 18:2n-6) and hydrogenated coconut oil (containing 12:0)-fed animals. The macrophage phospholipid PUFA profiles were compared with those of the liver, lung and spleen. The significance of the PUFA alterations is discussed.
Lipids 23, 380–383 (1988).

Depending upon the availability of membrane-bound fatty acids, determined largely by dietary constituents, cellular eicosanoid biosynthesis may be redirected in favor of a particular autacoid (1). For instance, it has been demonstrated that the oral administration of primrose oil, containing 9% γ-linolenic acid (18:3n-6), has the potential for increasing tissue biosynthesis of 1-series prostaglandins (PG_1) (2,3). This observation is of particular interest in view of the antiphlogistic properties of PG_1 (4,5). In addition, there are an impressive number of reports suggesting that dietary supplementation with fish oil, containing eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), may attenuate the phlogistic response (6,7). The efficacy of fish oil as a modulator of the immune system has been attributed to its content of n-3 fatty acids, which serve as precursors of cyclooxygenase and lipoxygenase metabolites (6,7).

Macrophages play a pivotal role in the amplification and suppression of inflammation and immune responses (8). These cells secrete prodigious quantities of arachidonic acid (20:4n-6)-derived eicosanoids (8). To further investigate the role of dietary manipulation involving 18:3n-6 and 20:5n-3 on macrophage phospholipids, mice were administered diets mixed with borage oil (BOR) containing 18:2n-6 and 18:3n-6, menhaden fish oil (MFO) containing 20:5n-3 and 22:6n-3, safflower oil (SAF) containing 18:2n-6, and hydrogenated coconut oil (HCO) containing 12:0. The polyunsaturated fatty acid (PUFA) profiles of macrophage total phospholipids were compared to those of the liver, lung and spleen.

MATERIALS AND METHODS

Pathogen-free female C57BL/6NCR mice (Charles River, Wilmington, MA), weighing 15–20 g were used. All mice had been fed a commercial pelleted mouse diet (Ralston-Purina, St. Louis, MO) and were housed (6–8 animals per cage) in autoclaved cages with laminar flow hoods in order to minimize spontaneous macrophage activation (9). On day zero of the experiment, the mice randomly were assigned to one of four purified diets for four wk, each adequate in all nutrients except 18:2n-6 (10). The diet varied only in the type of oil fed, i.e. either safflower, borage, fish or hydrogenated coconut oil made up 10% of the diet by weight. All dietary components were purchased from ICN Nutritional Biochemicals (Cleveland, OH), except where noted. The basic composition of the purified diet was casein, 20%; DL-methionine, 0.3%; corn starch, 15%; sucrose, 44%; cellulose, 5%; mineral mix, 3.5% (11); vitamin mix, 2% (11); choline chloride, 0.2%; and either SAF, 10% (California Oils, Richmond, CA); BOR, 10% (Traco Labs, Champaign, IL); MFO, 10% (Zapata-Haynie, Reedville, VA); or HCO, 10% (Capital City Products, Columbus, OH). The major fatty acids of the HCO diet were (in wt %) 8:0, 7.9%; 10:0, 6.9%; 12:0, 50.9%; 14:0, 18.2%; 16:0, 7.8%; 18:0, 7.1%; 18:1n-9, 1.0%; SAF diet, 16:0, 7.0%; 18:0, 2.5%; 18:1n-9, 10.7%; 18:2n-6, 78.2%: BOR diet, 16:0, 12.4%; 18:0, 4.4%; 18:1n-9, 19.6%; 18:2n-6, 36.6%; 18:3n-6, 25.2%; and MFO diet, 14:0, 7.6%; 16:0, 16.0%; 18:0, 2.8%; 18:1n-9, 10.5%; 18:2n-6, 1.2%; 18:3n-6, 0.6%; 20:4n-6, 0.6%; 20:5n-3, 16.9%; 22:5n-3, 2.5%; 22:6n-3, 12.0%. The diets were adequate in all nutrients, except HCO and MFO, which were deficient in 18:2n-6 (10). A diurnal light cycle of 12 hr was maintained, and food and autoclaved water were available ad libitum. The powdered diets were mixed and stored under N_2 at −20 C. Fresh diet was provided daily to each group to minimize auto-oxidation. Diets were checked periodicaliy by determination of the fatty acid composition. The profiles did not vary significantly in the diets over the four-wk feeding period. Body weights were recorded weekly, and the food intake of the SAF, BOR, MFO and HCO animals were diurnally monitored and not found to be significantly ($P > 0.05$) different (data not shown).

Macrophage cultures. Mice were injected intraperitoneally with 2 ml of 1.5% sterile sodium caseinate three days before being killed. Primary cultures of peritoneal macrophages were established from responsive cells as described previously (12).

Lipid analysis. Livers, spleens and lungs were excised, rinsed in ice-cold saline, flash frozen in liquid N_2 and held at −70 C for lipid extraction. Same-day macrophage cultures and frozen tissues were extracted (13), and the total phospholipids isolated by thin layer chromatography (14). Fatty acids were quantitated by gas chromatography (15). All data were subjected to one-way analysis of variance using multiple comparisons, with the upper level of significance chosen at $P < 0.05$.

RESULTS AND DISCUSSION

No differences between the diets were noted with respect to numbers or relative proportions of resultant peritoneal inflammatory exudate populations after sodium caseinate injection (data not shown). The data in Table 1 show that

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Abbreviations: BOR, borage oil; EFAD, essential fatty acid deficiency; HCO, hydrogenated coconut oil; MFO, menhaden fish oil; PUFA, polyunsaturated fatty acid; SAF, safflower oil.

TABLE 1

Polyunsaturated Fatty Acids in Macrophage Total Phospholipids

Fatty acid	HCO	SAF	BOR	MFO
18:2n-6	4.4 ± 0.1^{b}	11.6 ± 0.6^{a}	8.6 ± 0.1^{a}	3.6 ± 0.2^{b}
18:3n-6	tr^{a}	tr^{a}	tr^{a}	tr^{a}
20:3n-6	1.1 ± 0.0^{a}	1.0 ± 0.2^{a}	4.5 ± 0.1^{b}	0.4 ± 0.4^{a}
20:3n-9	1.4 ± 0.0^{b}	tr^{a}	tr^{a}	tr^{a}
20:4n-6	13.2 ± 1.0^{a}	15.0 ± 1.2^{a}	15.8 ± 1.0^{a}	6.3 ± 0.5^{b}
20:5n-3	tr^{a}	tr^{a}	tr^{a}	3.7 ± 0.7^{b}
22:4n-6	4.0 ± 0.1^{b}	7.9 ± 0.4^{d}	6.2 ± 0.7^{c}	tr^{a}
22:5n-6	0.8 ± 0.2^{a}	1.4 ± 0.1^{a}	1.3 ± 0.6^{a}	tr^{a}
22:5n-3	tr^{a}	tr^{a}	tr^{a}	6.8 ± 1.0^{b}
22:6n-3	tr^{a}	tr^{a}	tr^{a}	4.0 ± 0.4^{b}
20:3n-6/20:4n-6	0.08 ± 0.0^{a}	0.07 ± 0.02^{a}	0.28 ± 0.02^{b}	0.06 ± 0.06^{a}
20:5n-3/22:5n-3	<0.01^{a}	<0.01^{a}	<0.01^{a}	0.54 ± 0.05^{b}

Results are expressed in mg/100 mg total fatty acids present. Each figure represents the mean ±S.E.M. from pooled macrophage monolayers (n = 2).

Values with similar superscripts are not significantly different (P > 0.05).

Only selected polyunsaturated fatty acids are presented. tr, trace amounts, less than 0.1%.

TABLE 2

Polyunsaturated Fatty Acids in Liver Total Phospholipids

Fatty acid	HCO	SAF	BOR	MFO
18:2n-6	6.0 ± 0.4^{b}	19.4 ± 0.5^{d}	11.6 ± 0.3^{c}	2.6 ± 0.4^{a}
18:3n-6	tr^{a}	0.2 ± 0.1^{a}	1.6 ± 0.1^{b}	0.2 ± 0.0^{a}
20:3n-6	1.7 ± 0.2^{ab}	1.4 ± 0.1^{a}	2.4 ± 0.3^{b}	0.5 ± 0.0^{c}
20:3n-9	4.0 ± 0.5^{b}	tr^{a}	tr^{a}	tr^{a}
20:4n-6	13.0 ± 0.6^{b}	22.6 ± 0.1^{a}	23.8 ± 0.4^{a}	6.5 ± 0.1^{c}
20:5n-3	tr^{a}	tr^{a}	tr^{a}	9.4 ± 1.0^{b}
22:4n-6	tr^{a}	0.6 ± 0.0^{b}	0.8 ± 0.1^{b}	tr^{a}
22:5n-6	1.4 ± 0.1^{b}	3.7 ± 0.1^{c}	3.4 ± 0.2^{c}	tr^{a}
22:5n-3	tr^{a}	tr^{a}	tr^{a}	2.0 ± 0.0^{b}
22:6n-3	8.1 ± 0.5^{b}	5.5 ± 0.2^{a}	6.2 ± 0.3^{a}	24.2 ± 0.8^{c}
20:3n-6/20:4n-6	0.12 ± 0.01^{a}	0.06 ± 0.00^{b}	0.11 ± 0.01^{a}	0.08 ± 0.00^{b}
20:5n-3/22:5n-3	<0.01^{a}	<0.01^{a}	<0.01^{a}	4.52 ± 0.53^{b}

Results are expressed in mg/100 mg total fatty acids present. Each figure represents the mean ±S.E.M. (n = 3–4).

Values with similar superscripts are not significantly different (P > 0.05).

Only selected polyunsaturated fatty acids are presnted. tr, trace amounts, less than 0.1%.

TABLE 3

Polyunsaturated Fatty Acids in Spleen Total Phospholipids

Fatty acid	HCO	SAF	BOR	MFO
18:2n-6	3.0 ± 0.3^{a}	16.2 ± 1.6^{c}	6.8 ± 0.1^{b}	2.0 ± 0.3^{a}
18:3n-6	tr^{a}	0.1 ± 0.1^{a}	0.5 ± 0.2^{b}	0.1 ± 0.1^{a}
20:3n-6	1.1 ± 0.1^{b}	0.4 ± 0.2^{a}	3.4 ± 0.3^{c}	0.6 ± 0.1^{a}
20:3n-9	2.2 ± 0.8^{b}	tr^{a}	tr^{a}	tr^{a}
20:4n-6	19.1 ± 0.8^{a}	19.0 ± 1.1^{a}	21.4 ± 0.9^{a}	7.7 ± 0.4^{b}
20:5n-3	tr^{a}	tr^{a}	tr^{a}	8.5 ± 0.4^{b}
22:4n-6	1.9 ± 0.5^{b}	3.7 ± 0.2^{c}	4.2 ± 0.2^{c}	tr^{a}
22:5n-6	1.5 ± 0.1^{b}	2.3 ± 0.4^{b}	2.4 ± 0.4^{b}	tr^{a}
22:5n-3	tr^{a}	tr^{a}	tr^{a}	6.1 ± 0.1^{b}
22:6n-3	5.6 ± 0.6^{b}	1.8 ± 0.6^{a}	1.5 ± 0.9^{a}	10.6 ± 0.0^{c}
20:3n-6/20:4n-6	0.05 ± 0.0^{ab}	0.03 ± 0.02^{a}	0.18 ± 0.01^{c}	0.07 ± 0.01^{b}
20:5n-3/22:5n-3	<0.01^{a}	<0.01^{a}	<0.01^{a}	1.35 ± 0.07^{b}

Results are expressed in mg/100 mg total fatty acids present. Each figure represents the mean ±S.E.M. (n = 4).

Values with similar superscripts are not significantly different (P > 0.05).

Only selected polyunsaturated fatty acids are presented. tr, trace amounts, less than 0.1%.

TABLE 4

Polyunsaturated Fatty Acids in Lung Total Phospholipids

Fatty acid	HCO	SAF	BOR	MFO
18:2n-6	2.4 ± 0.5[a]	9.8 ± 0.6[c]	5.2 ± 0.3[b]	1.6 ± 0.1[a]
18:3n-6	tr[a]	tr[a]	0.9 ± 0.0[b]	0.1 ± 0.0[a]
20:3n-6	0.7 ± 0.0[a]	0.8 ± 0.0[a]	2.3 ± 0.3[b]	0.4 ± 0.0[a]
20:3n-9	1.4 ± 0.4[b]	tr[a]	tr[a]	tr[a]
20:4n-6	9.2 ± 1.1[b]	11.1 ± 0.9[b,c]	12.4 ± 0.5[c]	5.8 ± 0.7[a]
22:4n-6	0.8 ± 0.5[a]	3.5 ± 0.4[b]	1.8 ± 0.3[b]	0.2 ± 0.1[a]
22:5n-6	0.5 ± 0.2[a,b]	1.4 ± 0.4[a,b]	1.8 ± 0.3[b]	0.2 ± 0.1[a]
20:5n-3	tr[a]	tr[a]	tr[a]	5.8 ± 2.3[b]
22:5n-3	0.4 ± 0.2[a]	tr[a]	tr[a]	3.1 ± 1.0[b]
22:6n-3	5.4 ± 0.8[a]	2.0 ± 0.3[a]	1.7 ± 0.2[a]	13.5 ± 2.9[b]
20:3n-6/20:4n-6	0.08 ± 0.01[a]	0.07 ± 0.01[a]	0.18 ± 0.02[b]	0.08 ± 0.00[a]
20:5n-3/22:5n-3	<0.01[a]	<0.01[a]	<0.01[a]	2.12 ± 1.42[b]

Results are expressed in mg/100 mg total fatty acids present. Each figure represents the mean ±S.E.M. (n = 3).
Values with similar superscripts are not significantly different (P > 0.05).
Only selected polyunsaturated fatty acids are presented. tr, trace amounts, less than 0.1%.

the n-3 fatty acids, 20:5n-3 and 22:6n-3, were readily incorporated into macrophage phospholipids in MFO-fed animals. Interestingly, 18:3n-6 was not detected in BOR macrophages, which contrasted with liver (Table 2), spleen (Table 3) and lung (Table 4) phospholipids, in which 18:3n-6 levels were significantly elevated ($P < 0.05$) relative to HCO-, SAF- and MFO-fed mice. In addition, the macrophage 20:3n-6/20:4n-6 ratio was elevated significantly ($P < 0.05$) in BOR animals. This ratio was highest in the macrophage (0.28 ± 0.02) relative to the liver (0.11 ± 0.01), spleen (0.18 ± 0.01) and lung (0.18 ± 0.02). These results suggest that the macrophage is capable of elongating 18:3n-6 into 20:3n-6 and that the Δ5 desaturase, the enzyme that catalyzes the transformation of 20:3n-6 into 20:4n-6, is rate-limiting. This scenerio is consistent with our earlier findings (16), in which the presence of a PUFA chain elongase in mouse peritoneal macrophages in vitro was noted. The striking elevation of BOR 20:3n-6/20:4n-6 is of particular interest because 20:3n-6 can serve as a precursor for the biosynthesis and increase of 1-series prostaglandins. These monoenoic eicosanoids possess anti-inflammatory properties (4,5).

The accumulation of 20:3n-9 and the depression of 18:2n-6 in HCO tissues is indicative of essential fatty acid deficiency (EFAD) (17). Of the cells or organs examined, the 20:3n-9/20:4n-6 ratio was greatest in the liver. The significant depression ($P < 0.05$) of macrophage 18:2n-6 and 20:4n-6 in MFO-fed mice as shown in Table 1 is also indicative of EFAD. This finding is in accord with the results of Lokesh et al. (18).

Only mice receiving MFO had significant levels of docosapentaenoic acid (22:5n-3) in the phospholipid pool. Since only small quantities of 22:5n-3 are found in the MFO diet, it is likely that this n-3 fatty acid was derived via chain elongation of 20:5n-3. Interestingly, in MFO-fed mice, the 20:5n-3/22:5n-3 ratio was lowest in the macrophage (0.54 ± 0.05) relative to liver (4.52 ± 0.53), spleen (1.35 ± 0.07) and lung (2.12 ± 1.42). These data do not provide definitive proof but suggest, as mentioned above, that macrophages possess an active long chain PUFA chain elongase. Consistent with these observations are the results of Magrum and Johnston (19), who similarly reported an increase in rat peritoneal macrophage phospholipid 22:5n-3 levels after feeding an n-3 fatty acid rich diet. Although it has been demonstrated that dietary MFO is capable of inhibiting the conversion of 20:4n-6 into pro-inflammatory eicosanoids in the macrophage (18,20), further work is needed to determine whether n-3 fatty acid incorporation alters macrophage functional properties, i.e. cytolytic capacity.

In conclusion, the PUFA composition of macrophage phospholipids can be manipulated in vivo by administering specific dietary oils. Because the macrophage plays a central role in the immune system, further work is needed to fully evaluate the effects of borage and fish oil feeding on the mechanisms of signal transduction and production of regulatory factors by this complex cell type.

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REFERENCES

1. Mathias, M.M., and Dupont, J. (1985) *Lipids 20,* 791-801.
2. Kunkel, S.L., Ogawa, H., Ward, P.A., and Zurier, R.B. (1981) *Prog. Lipid Res. 20,* 885-889.
3. Zurier, R.B. (1982) in *Clinical Uses of Essential Fatty Acids* (Horrobin, D.F., ed.) pp. 113-124, Eden Press, Montreal, Canada.
4. Kunkel, S.L., Thrall, R.S., Kunkel, R.G., McCormick, J.R., Ward, P.A., and Zurier, R.B. (1979) *J. Clin. Invest. 64,* 1525-1529.
5. Zurier, R.B., and Quagliata, F. (1971) *Nature 234,* 304-305.
6. Terano, T., Salmon, J.A., Higgs, G.A., and Moncada, S. (1986) *Biochem. Pharmacol. 35,* 779-785.
7. Lee, T.H., Mencia-Huerta, J.M., Shih, C., Corey, E.J., Lewis, R.A., and Austen, K.F. (1984) *J. Biol. Chem. 259,* 2283-2387.
8. Nathan, C.F., and Cohn, Z.A. (1985) in *Textbook of Rheumatology* (Kelley, W.W., Harris, E.D., Ruddy, S., and Sledge, C.B., eds.) pp. 144-169, W.B. Saunders, Philadelphia, PA.

9. Meltzer, M.S. (1976) *Cell Immunol. 75,* 176–186.
10. National Research Council (1978) in *Nutrient Requirements of Laboratory Animals,* Vol. 10, pp. 38–53, National Academy of Sciences, Washington, D.C.
11. Erickson, K.L., Schlanger, D.S., Adams, D.A., Fregeau, D.R., and Stern, J.S. (1984) *J. Nutr. 114,* 1834–1842.
12. Bockman, R.S. (1981) *Prostaglandins 21,* 9–31.
13. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) *J. Biol. Chem. 22,* 497–509.
14. Chapkin, R.S., Ziboh, V.A., and McCullough, J.L. (1987) *J. Nutr. 117,* 1360–1370.
15. Chapkin, R.S., Ziboh, V.A., Marcelo, C.L., and Voorhees, J.J. (1986) *J. Lipid Res. 27,* 945–954.
16. Chapkin, R.S., Somers, S.D., and Erickson, K.L. (1987) in *Polyunsaturated Fats and Eicosanoids* (Lands, W.E.M., ed.), American Oil Chemists' Society, pp. 395–398.
17. Holman, R.T. (1971) *Prog. Lipid Res. 9,* 275–348.
18. Lokesh, B.R., Hsieh, H.L., and Kinsella, J.E. (1986) *J. Nutr. 116,* 2547–2552.
19. Magrum, L.J., and Johnston, P.V. (1983) *Lipids 18,* 514–521.
20. Leslie, C.A., Gonnerman, W.A., Ullman, M.D., Hayes, K.C., Franzblau, C., and Cathcart, E.S. (1985) *J. Exp. Med. 162,* 1336–1349.

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ERRATUM

Portions of the text in "Accumulation of an Alkyl Lysophospholipid in Tumor Cell Membranes Affects Membrane Fluidity and Tumor Cell Invasion" by W. J. van Blitterswijk et al. (*Lipids 22*, 820–823) were inadvertently misplaced. The paragraph beginning "In these experiments . . ." in the second column of page 820 and the following two paragraphs of text should have been published at the end of the Results and Discussion section.

Membrane Lipid Alteration: Effect on Cellular Uptake of Mitoxantrone

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We have studied the effect of membrane structural alteration on the cellular association of the anticancer drug mitoxantrone whose uptake is not carrier-mediated. Membrane fatty acids of L1210 cells were modified by incubating the cells with the highly unsaturated docosahexaenoic acid (22:6), which results in isolated plasma membranes with 37% of the fatty acids as 22:6, or with the monounsaturated oleic acid (18:1), which results in 58% of the fatty acids as 18:1. The rate of uptake by 22:6-enriched cells during the first min was 62% greater than by those enriched with 18:1. The higher rate was recorded at 0.5–16 μM, pH 6.6–7.6 and temperatures 10–40 C. The difference in cell-associated drug apparently was not due simply to a change in mitoxantrone solubility as measured by partitioning of the drug in lipophilic-hydrophilic systems containing lipids from the fatty-acid altered cells. We conclude that the type of fatty acids contained in L1210 cell membranes can affect the cell association of mitoxantrone. This effect could be on transmembrane flux or be due to differences in binding of the drug to intracellular structures.
Lipids 23, **393–397 (1988).**

It has been shown that alteration of fatty acids affects permeation in artificial membrane systems (1). It also has been demonstrated in biological systems that alteration in fatty acids influences carrier-mediated transport (2). However, it is unclear whether lipid alteration can affect simple diffusion through cellular membranes. We recently have reported that the unidirectional influx and efflux of mitoxantrone or 1,4-dihydroxyl-5,8-bis[[2-[(2-hydroxyethyl) amino]ethyl]amino]-9,10-anthracenedione dihydrochloride in murine leukemia cells occur by passive diffusion (3). Mitoxantrone is an investigational antitumor drug with reported activity in advanced breast cancer, lymphomas and acute leukemias. There is considerable information on its chemistry (4,5), mechanism of action (6–8) and pharmacokinetics (9). Attempting to understand better the role of membrane lipids in diffusion, we studied mitoxantrone uptake by L1210 cells whose membrane lipids had been altered experimentally. For these studies, we utilized a method previously reported by us for altering the types of lipids in the membranes of L1210 leukemia cells (10). This type of alteration results in a change in membrane molecular order as measured by electron spin resonance (11) and in transport of compounds whose transmembrane movement is governed by a carrier-mediated process (12–15). We report here that fatty acid alteration influences the cell association of a molecule that requires neither a carrier nor expenditure of energy.

MATERIALS AND METHODS

L1210 murine leukemia cells were grown and maintained in suspension culture at 37 C in medium consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), 5% fetal bovine serum (Hazleton Research Products, Denver, PA) and gentamicin sulfate (50 μg/ml) in a humid atmosphere of 5% CO_2/95% air. These cells originally were obtained from David Vistica of the National Institutes of Health. Cells were harvested during exponential growth phase and resuspended in balanced salt solution (BSS: 132 mM NaCl, 16 mM Na_2PO_4, 5 mM KCl, 1 mM $MgSO_4$, 5.6 mM glucose, pH 7.4) for transport studies.

[^{14}C]mitoxantrone, labeled in the carbon atoms α to the arylamine groups, was supplied by B. M. Silber, American Cyanamid Company (Pearle River, NY). It was received as a powder (40 Ci/mol), was constituted with distilled water and then frozen at −70 C in aliquots until use. Purity after thawing and just before use was 99% as determined in our laboratory using high performance liquid chromatography (16).

Permeability studies were performed by modification of previously described techniques from our laboratory (3,13,17,18). Studies were carried out in small vessels containing 1×10^6 cells in 0.5 ml BSS. After temperature equilibration, [^{14}C]mitoxantrone was added. At the termination of incubation, the cell suspension was transferred to 1.5 ml tapered Eppendorf microcentrifuge tubes at 0 C containing 0.25 ml n-butyl phthalate/corn oil (2.75:1, v/v) layered beneath 0.3 ml BSS. Rapid separation of the cells from incubation medium was accomplished by immediate centrifugation at 15600 × g for 30 sec in an Eppendorf 5412 centrifuge. The supernatant then was aspirated, and the tip of the tapered tube containing the cell pellet was severed and placed in a scintillation vial containing 0.5 ml NCS tissue solubilizer (Amersham, Arlington Heights, IL). After 60 min, Neutralizer scintillation mixture (Research Products International, Elk Grove Village, IL) was added and radioactivity determined using a Beckman LS3133T scintillation spectrometer. Drug uptake at each concentration was corrected for rapid association with the plasma membrane except where indicated in the time course studies. It was estimated by measuring cell association of [^{14}C]mitoxantrone at 0 C in a time interval of less than three sec. Temperature dependence of transport was determined at 2 C intervals from 10 C to 40 C. Incubations were for 45 sec in the presence of 8 μM mitoxantrone following a five-min equilibrium period for the cells and medium at the respective temperature. Efflux studies were performed using L1210 cells pre-loaded with [^{14}C]mitoxantrone (4 μM, 30 min, 37 C), which then were rapidly chilled and washed at 0 C. Cells were resuspended in balanced salt solution at 37 C to initiate efflux.

To modify the cellular and membrane fatty acid composition, cells were grown for two days under identical conditions in medium further supplemented with either oleic acid (18:1) or docosahexaenoic acid (22:6) (NuChek Prep, Inc., Elysian, MN) (fatty acids are abbreviated as number of carbon atoms:number of double bonds). Specific quantities of sodium salts of each fatty acid were added dropwise to the fetal bovine serum used to prepare the growth medium so that their concentration in the final growth medium was 32 μM. Cellular protein was determined (19). Those cells modified with 22:6 contained

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138.39 ± 13.29 μg protein per 10^6 cells (n = 9), and this was not significantly different from the 18:1 modified cells that contained 151.79 ± 18.75 μg.

Octanol partitioning of the drug was measured by dissolving 2.5 μg (4.25 × 10^5 dpm) of [^{14}C]mitoxantrone in 500 μl of phosphate-buffered saline (NaCl 136.8 mM, Na_2HPO_4 8.06 mM, KCL 2.68 mM, KH_2PO_4 1.47 mM, $CaCl_2$ 0.9 mM, $MgCl_2$ 0.6 mM) at pH 7.2 previously saturated with octanol. An equal volume of octanol previously saturated with phosphate-buffered saline was added. After mixing, the phases were separated by centrifugation at 300 × g and the radioactivity in each phase determined (20). The study was repeated in the presence of lipid extracts from 18:1- and 22:6-enriched cells using 1.5 × 10^7 cells.

Partitioning of the radiolabeled drug into lipid-containing compartments to measure interaction of drug with lipids was assayed by a modified Folch-type extraction (21). L1210 cells (1 × 10^7) enriched in 22:6 or 18:1 were extracted with $CHCl_3/CH_3OH$ (2:1) and 2.5 μg [^{14}C]mitoxantrone (4.2 × 10^5 dpm) was added. The mixture was shaken, and 5 ml of 0.04 N KCl were added, mixed and the phases allowed to separate overnight. The separate phases were collected, dried under N_2, solubilized with NCS tissue solubilizer, and the radioactivity determined.

To prepare purified plasma membranes, L1210 cells grown in medium supplemented with either 22:6 or 18:1 were washed and disrupted as described (13). Subcellular fractions were separated using two discontinuous sucrose gradients (22,23). 5′-Nucleotidase (E.C. 3.1.3.5) activity as a plasma membrane marker, was determined on each fraction in each experiment (24), and the plasma membrane fraction collected at the 10%–30% interface was enriched an average of 12-fold as compared with the homogenate.

The plasma membrane fractions from the discontinuous gradients for 18:1- and 22:6-modified cells were extracted using $CHCl_3/CH_3OH$ (2:1, v/v) (25). The lipids then were saponified for 60 min at 56 C in 1.2 N KOH and 80% ethanol (26). Fatty acids in the saponifiable fraction were methylated for 10 min at 95% with 14% BF_3/CH_3OH (27), and the methyl esters were separated by gas chromatography using a 1.8 m column packed with 10% SP2340 on 100/120 mesh Chromosorb (Supelco, Inc., Bellefonte, PA). Peak areas were quantitated and identified by comparison of retention times to those of standards obtained from Supelco.

RESULTS

Lipid analysis. To ascertain the extent of fatty acid modification that occurred in the plasma membranes of the cells grown in lipid-supplemented media, the fatty acid composition of the purified membrane fractions was determined. There was considerable enrichment of the membranes with the fatty acid supplemented in the medium (Table 1). The membranes from the 18:1-enriched cells contained 61% monoenic fatty acids, the majority (59%) being 18:1. The membranes from the 22:6-enriched cells contained 44% polyenoic fatty acids; 37% of the fatty acids were 22:6. The 22:6 membranes contained more than two-fold greater proportion of double bonds.

Time course of mitoxantrone uptake. The uptake of mitoxantrone by both 18:1-modified and 22:6-modified L1210 cells was studied at nine time points between 15 sec and 30 min (Fig. 1). Initial uptake was linear for 60 sec and then gradually slowed to reach a plateau by about 20 min. The rate of cell association during the initial 60 sec was greater by the 22:6-enriched cells (slope = 91.7 ± 8.6) than by the 18:1-enriched cells (slope = 56.5 ± 11.3) ($p = 0.03$, n = 5). The rapid association of drug with the plasma membrane measured experimentally at 0 C was not significantly different between the two modified cell types. The differences in the accumulation of drug disappeared at later time points (Fig. 1, insert).

Concentration dependence of mitoxantrone uptake. The concentration dependence of drug uptake for both types of enriched cells was linear for a drug concentration range 0.5–16 μM. The amount of drug taken up was higher by the 22:6-enriched cells at all seven concentrations, and this difference was statistically significant at all but the

TABLE 1

Fatty Acid Composition of L1210 Plasma Membranes[a]

Fatty acid	Fatty acid composition (%)	
	22:6-Supplemented	18:1-Supplemented
Individual acids		
16:0[b]	14.4 ± 2.0[c]	11.7 ± 1.7
16:1	ND[d]	2.0 ± 0.6
18:0	18.7 ± 0.8	13.0 ± 1.2
18:1	21.8 ± 0.8	58.8 ± 1.5
18:2	0.2 ± 0.1	0.5 ± 0.3
18:3	0.3 ± 0.3	3.9 ± 0.3
20:4	3.7 ± 0.3	5.2 ± 0.5
20:5	1.3 ± 0.1	0.4 ± 0.2
22:5	1.4 ± 0.5	2.4 ± 0.4
22:6	37.4 ± 3.6	1.5 ± 0.2
Other[e]	0.8	0.6
Classes		
Saturates	33.6 ± 2.6[f]	25.0 ± 2.1
Monoenoics	21.8 ± 0.8	60.7 ± 1.3
Polyenoics	44.4 ± 3.3	14.1 ± 1.1
Mean no. double bonds	2.8 ± 0.2	1.2 ± <0.1
Mean chain length	19.3 ± 0.2	18.0 ± <0.1

[a]L1210 cells were grown for 48 hr in RPMI 1640 supplemented with either 22:6 or 18:1 at a concentration of 32 μM. Cells were washed three times, mechanically disrupted and subcellular fractions prepared by gradient centrifugation. Lipids extracted from the membranes were subjected to alkaline hydrolysis. Fatty acids contained in the saponifiable fraction were methylated and the methyl esters separated by gas liquid chromatography.

[b]The fatty acids are signified as number of carbon atoms: number of double bonds.

[c]Mean ± S.E. of determinations made on individual membrane samples shown are from three to four separate experiments. Among the individual acids, the amounts of 18:1, 18:3, and 22:6 ($p < 0.001$), 18:0, 20:5 and 20:4 ($p < 0.05$) are significantly different between the supplemented cell membranes.

[d]ND, not detected.

[e] Includes small amounts of 14:0, 20:1, 20:3, 22:1, 22:4 and unidentified fatty acids.

[f]Among the classes of fatty acids, the amounts of polyenoics, monoenoics, average number of double bonds ($p < 0.001$), average chain length and saturates ($p < 0.05$) are significantly different between the supplemented cell membranes.

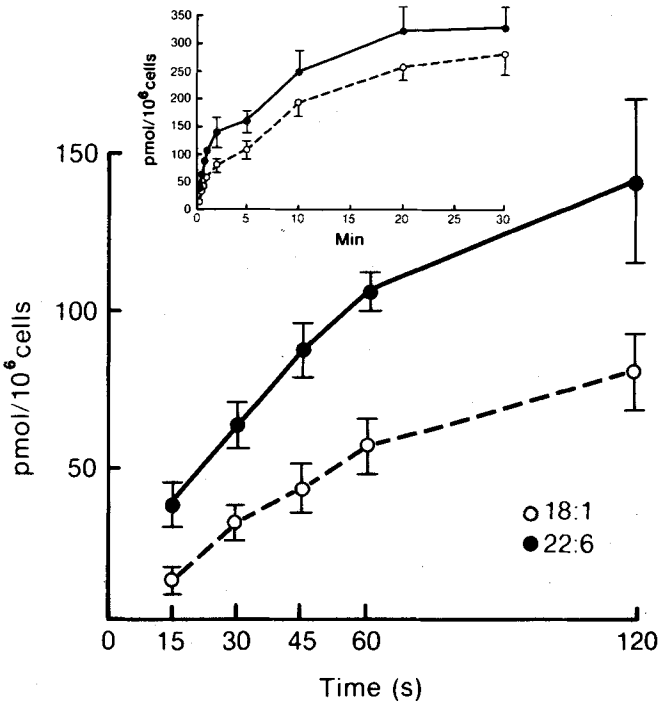

FIG. 1. Time course of mitoxantrone uptake by fatty acid-modified L1210 cells. L1210 cells enriched with 22:6 or 18:1 were incubated at 37 C with 4 μM [^{14}C]mitoxantrone in balanced salt solution. At the termination of the incubation, the cells were rapidly centrifuged through an oil layer, and the radioactivity of the cell pellet was determined. Each point represents the mean ± S.E. of at least four determinations. Insert shows uptake at later time points.

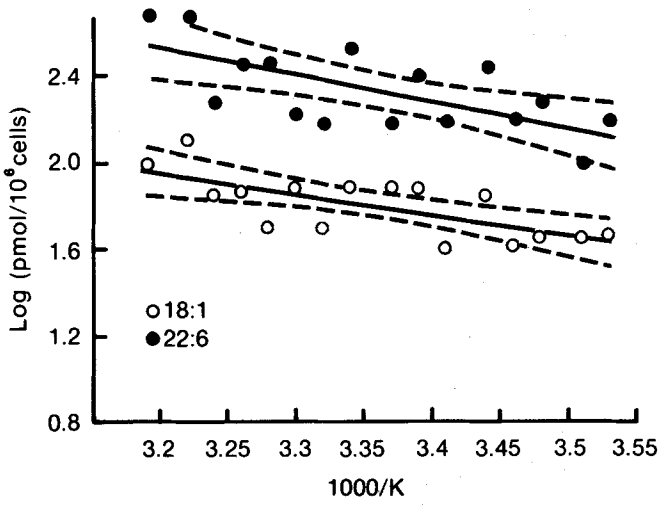

FIG. 2. Arrhenius plot of temperature dependence of mitoxantrone transport. Fatty acid modified L1210 cells were incubated with 8 μM [^{14}C]mitoxantrone for 45 sec at 2 C intervals from 10–40 C. Cells were rapidly centrifuged through an oil layer, and radioactivity in the pellet was determined. Shown are the mean of three experiments and the 95% confidence limits.

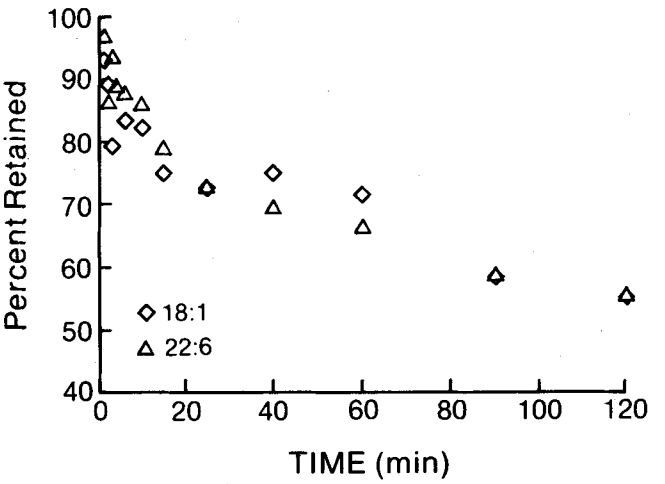

FIG. 3. Efflux of mitoxantrone by fatty acid modified cells. L1210 cells were incubated with 4 μM [^{14}C]mitoxantrone for 30 min at 37 C. Cells were chilled, washed at 0 C and efflux measured by determining intracellular radioactivity at specified times.

lower concentrations. When the slopes of the linear regression lines (for 18:1, $y = 24.53x - 0.18$, $r = 0.99$; for 22:6, $y = 36.74x - 7.39$, $r = 0.99$) were compared, that of the 22:6-enriched cells was greater than the 18:1-enriched cells ($p = 0.008$).

Effect of temperature and pH. Both 18:1- and 22:6-enriched L1210 cells demonstrated limited temperature dependence for initial mitoxantrone cell association. Three separate experiments were performed, during which the initial uptake during 45 sec of 8 μM drug was studied at 2 C-intervals from 10–40 C. All studies demonstrated an increased uptake with increasing temperature (Fig. 2). Although the relationship appeared to be roughly linear ($r = 0.712$ for 18:1, $p = 0.002$ and $r = 0.657$ for 22:6, $p = 0.006$), the noise in the data precludes identification of break points. If linearity is assumed, the slope for the 18:1-enriched cells (−0.012 ± <0.001) was not different from the slope for the 22:6-enriched cells (−0.012 ± 0.003). However, the uptake of drug by the 22:6-enriched cells was higher at all temperatures. At each of the four pH values tested between 6.6 and 7.7, the 22:6-enriched cells had a higher rate of influx than the 18:1-enriched cells.

Efflux. We next examined the rate of efflux of mitoxantrone from 18:1- and 22:6-enriched cells that were preloaded with drug. Initial efflux was rapid and linear for both cell types during the first 15 min with a subsequent decrease during the next 105 min (Fig. 3). Both reached a plateau of about 55% retention at 120 min. Neither the rate of efflux nor the plateau level were different for the cell lines. The values could represent the kinetics of efflux through the membrane or dissociation of drug from a binding site. The decline of cell associated drug to only 50% of the amount when loaded suggests there is a major binding component in either case.

Drug partitioning experiments. The octanol:water partition results demonstrated that mitoxantrone is a fairly lipophilic drug. A majority of the drug partitioned in the octanol (85% ± 1, n = 4) and the partition ratio (ratio of drug in octanol to that in aqueous phase) was 6.1 ± 0.7. The presence of lipids extracted from 22:6- or 18:1-enriched cells resulted in a decrease in partition ratio (18:1, 2.99 ± 0.18 vs 22:6, 2.88 ± 0.41), and in percentage of drug partitioning into octanol (18:1, 75.30% ± 1.55

TABLE 2

Effect of L1210 Lipids Extracted from 22:6- and 18:1-Enriched Cells on Partitioning Between Lipophilic and Hydrophilic Phases in a Folch Biphasic System

	Percentage of [^{14}C]mitoxantrone		
	Lipophilic	Hydrophilic	Interface
22:6	85.4 ± 3.3	9.6 ± 3.7	5.0 ± 1.8
18:1	81.8 ± 0.6	13.3 ± 2.2	5.1 ± 1.7
No cells	4.2 ± 0.4	95.8 ± 0.5	—

L1210 cells enriched with 22:6 or 18:1 were extracted with chloroform/methanol (2:1, v/v) and [^{14}C]mitoxantrone added. To separate the layers, 0.04 N KCl was added, mixed, and the phases allowed to separate. The separate phases were then collected, dried under N_2, solubilized, and the radioactivity determined. Values are the mean and S.E. of four replicates.

vs 22:6, 73.40% ± 2.66) but were not different between lipids from the two types of modified cells.

We also compared the physical association of mitoxantrone with the lipids extracted from modified cells using a Folch extraction. L1210 cells enriched with either 22:6 or 18:1 were lipid extracted using chloroform/methanol (2:1, v/v). We have shown that there was no difference in total acylglycerols, phospholipids or cholesterol between L1210 cells enriched with 22:6 or 18:1 (2,11). [^{14}C]Mitoxantrone was added, and the phases separated by the addition of 0.04 N KCl. The lower lipophilic phase, the upper hydrophilic phase and the protein-rich precipitate at the interface were separated and the radioactivity in each determined. Table 2 shows the proportion of drug partitioning into each phase. The majority of the drug partitioned into the lipophilic phase. Most importantly, there was no difference in the proportion of drug in the lipophilic layer extracted from 22:6 vs 18:1 cells. This shows that there is no differential complexing of mitoxantrone with lipids in vitro. This is consistent with relative octanol partitioning, which likewise showed no difference. It is interesting that the majority of the drug partitioned into the hydrophilic phase when no cell extract was present; however, the presence of lipids resulted in the partitioning of drug into the lipophilic phase.

DISCUSSION

These results indicate that L1210 cells that have enrichment of their membrane phospholipids with polyunsaturated fatty acids have a more rapid rate of cell-association of the anticancer drug mitoxantrone as compared with L1210 cells enriched with monoenoic fatty acids. The higher rate of association is time and drug concentration dependent. The difference was apparent at all temperatures and all medium pH-values studied. The observations of the present study are important because mitoxantrone uptake is not mediated by a membrane carrier nor does its influx require energy expenditure. Data from our laboratory and others have indicated that a number of transport systems of murine and human tumor cells are affected by the types of fatty acid modification that can be produced. For example, the apparent Km for high-affinity transport of the anticancer drug methotrexate (13), α-aminoisobutyrate (12), choline (14) and taurine (15) is lowered when the cells are enriched with polyunsaturated fatty acids. In each of those cases, the transport process is carrier-mediated, and the effect probably results from changes in binding of the substrate to the carrier due to a lipid-mediated change in conformation of the membrane carrier molecule. We have reported that fatty acid alteration affects the accumulation of Adriamycin by L1210 cells (18), a drug whose permeation mechanism has not been definitely characterized yet (18,28–30). However, we have reported that mitoxantrone enters cells by passive diffusion (3). Therefore, the observation that a lipid structural change influences its cell-association indicates that the effect is a result of changes in the lipids themselves and the interaction of the anticancer drug with the membrane lipids. The uniqueness of the study is the observation that the lipids may affect permeation directly rather than through an interaction with a membrane-based carrier molecule or by an effect on an energy requiring or potential-driven reaction.

The efflux studies indicate that there is a considerable amount of binding of drug to intracellular structures. It is possible that the rates of uptake measured in our kinetic studies are rates of binding to intracellular components rather than unidirectional influx. The apparently concentrative accumulation of drug even at early time points raises the possibility that the rates being measured are those of binding. In this regard, it has been reported that aminoanthraquinones bind to two distinct low molecular weight intracellular macromolecular lipids in L1210 cells (31). However, the fact that the process is nonsaturable up to 1 mM at both 0 and 37 C and that there is an almost two-fold difference in cell-association between 37 C and 0 C would be more compatible with unidirectional influx (3). We cannot distinguish with assurity whether the lipid alteration is influencing nonmediated permeation or intracellular binding.

No studies of the parent unmodified L1210 cell line were performed since we feel that such data could be misleading if compared with modified cells. Any differences or similarities found in a comparison of unmodified and lipid-modified cells could be ascribed to exposure of cells to fatty acids in the media per se rather than fatty acid modification. It was our intention to compare the effect of alteration of membrane fatty acids of diverse chemical and physical properties. The 18:1-modified cells in this sense act as a control to study the effect of enrichment with polyunsaturated fatty acids. Furthermore, the fatty acid composition of 18:1-enriched cells is similar to that of unmodified cells (11). We reported the mitoxantrone uptake characteristics of the parent line (3).

The mechanism by which fatty acid modification alters the uptake of mitoxantrone is unclear. The dissimilar uptake by the lipid modified cells could be related to a difference in membrane order. Current concepts of the mechanisms of diffusional permeation of substances across the membranes of isolated cells have been reviewed recently (32). We reported that in vitro modification of L1210 leukemia membrane lipids with 22:6 results in a highly significant decrease in the order parameter of an electron spin resonance probe (11). The molecular basis of diffusion and the relationship of membrane order and permeability is complex (32,33), but it is likely that the

permeation of mitoxantrone proceeds more rapidly through 22:6-enriched membranes as a result of a change in physicochemical properties. The octanol partitioning experiments demonstrated no difference in drug solubility in membranes enriched in the fatty acids. However, the partitioning across a single homogeneous phase boundary as measured is likely to be a poor model for membranes in an aqueous environment. We cannot rule out the possibility of differences in the unstirred layer or in thickness of the plasma membrane induced by fatty acid modification. However, these possibilities seem considerably less likely to explain mitoxantrone flux differences as compared with the change in membrane order. If a component of measured uptake represents binding, a possibility discussed above, then the altered lipids represent a plausible site for such binding. Experimental modification of a binding site with resulting change in binding affinity would provide a possible explanation for differences in cell-association.

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REFERENCES

1. Demel, R.A., Guerts van Kessel, W.S.M., and van Deenen, L.L.M. (1972) *Biochim. Biophys. Acta 266,* 26-40.
2. Burns, C.P., and Spector, A.A. (1987) *Lipids 22,* 178-184.
3. Burns, C.P., Haugstad, B.N., and North, J.A. (1987) *Biochem. Pharmacol. 36,* 857-860.
4. Zee-Cheng, R.K.Y., and Cheng, C.C. (1978) *J. Med. Chem. 21,* 291-294.
5. Murdock, K.C., Child, R.G., Fabio, P.F., Angier, R.B., Wallace, R.E., Burr, F.E., and Citarella, R.V. (1979) *J. Med. Chem. 22,* 1024-1030.
6. Kapuscinski, J., Darzynkiewicz, Z., Traganos, F., and Melamed, M.R. (1981) *Biochem. Pharm. 30,* 231-240.
7. Bowden, G.T., Roberts, R., Alberts, D.S., Peng, Y., and Garcia, D. (1985) *Cancer Res. 45,* 4915-4920.
8. Kapuscinski, J., and Darzynkiewicz, Z. (1986) *Proc. Natl. Acad. Sci. USA 83,* 6302-6306.
9. Alberts, D.S., Peng, Y.M., Bowden, G.T., Dalton, W.S., and Mackel, C. (1985) *Inv. New Drugs 3,* 101-107.
10. Guffy, M.M., North, J.A., and Burns, C.P. (1984) *Cancer Res. 44,* 1863-1866.
11. Guffy, M.M., Rosenberger, J.A., Simon, I., and Burns, C.P. (1982) *Cancer Res. 42,* 3625-3630.
12. Kaduce, T.L., Awad, A.B., Fontenelle, L.J., and Spector, A.A. (1977) *J. Biol. Chem. 252,* 6624-6630.
13. Burns, C.P., Luttenegger, D.G., Dudley, D.T., Buettner, G.R., and Spector, A.A. (1979) *Cancer Res. 39,* 1726-1732.
14. Hyman, B.T., and Spector, A.A. (1982) *J. Neurochem. 38,* 650-656.
15. Yorek, M.A., Strom, D.K., and Spector, A.A. (1984) *J. Neurochem. 42,* 254-261.
16. Peng, Y.M., Ormberg, D., Alberts, D.S., and Davis, T.P. (1982) *J. Chromatogr. 233,* 235-247.
17. Burns, C.P., and Dudley, D.T. (1982) *Biochem. Pharmacol. 31,* 2116-2119.
18. Burns, C.P., and North, J.A. (1986) *Biochim. Biophys. Acta 888,* 10-17.
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
20. Kessel, D. (1979) *Biochem. Pharm. 28,* 3028-3030.
21. Duarte-Karim, M., Ruysschaert, J.M., and Hildebrand, J. (1976) *Biochem. Biophys. Res. Commun. 71,* 658-663.
22. Tsai, C-M., Chen, K-Y., and Canellakis, E.S. (1975) *Biochim. Biophys. Acta 401,* 196-212.
23. Yang, C.H., Peterson, R.H.F., Sirotnak, F.M., and Chello, P.L. (1979) *J. Biol. Chem. 254,* 1402-1407.
24. Avruch, J., and Wallach, D.F.H. (1971) *Biochim. Biophys. Acta 233,* 334-347.
25. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
26. Abell, L.L., Levy, B.B., Brodie, B.B., and Kendall, F.E. (1952) *J. Biol. Chem. 195,* 357-366.
27. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600-608.
28. Skovsgaard, T., and Nissen, N.I. (1982) *Pharmac. Ther. 18,* 293-311.
29. Siegfried, J.M., Burke, T.G., and Tritton, T.R. (1985) *Biochem. Pharm. 34,* 593-598.
30. Johnson, B.A., Cheang, M.S., and Goldenberg, G.J. (1986) *Cancer Res. 46,* 218-223.
31. Taylor, R.F., Teague, L.A., and Yesair, D.W. (1981) *Cancer Res. 41,* 4316-4323.
32. Schafer, J.A. and Barfuss, D.W. (1986) in *Membrane Transport of Antineoplastic Agents* (Goldman, I.D., ed.), pp. 1-37, Pergamon Press, Oxford.
33. Lieb, W.R., and Stein, W.D. (1986) *J. Membrane Biol. 92,* 111-119.

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Molecular Species of Glycerolipids of Ehrlich Ascites Cells and of Their Fat Granules

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Ehrlich ascites cells were grown in mice and were isolated by centrifugation of the ascites fluid. The cells were lysed with distilled water, and the floating fat particles were collected by centrifugation. The particles contained about 90% neutral and 10% polar lipid. The neutral lipid was made up of about 50% triacylglycerol, 30% alkyldiacylglycerol, 3% cholesteryl esters, 3% free cholesterol and 4% free diacylglycerols. The phospholipid fraction was comprised of about 50% phosphatidylcholine, 35% phosphatidylethanolamine, 10% sphingomyelin and small amounts (less than 5% total) of serine and/or inositol phosphatides. The triacylglycerol and alkyldiacylglycerol fractions possessed total carbon number and fatty acid compositions closely similar to those reported in the literature for whole ascites cells and for a cell membrane preparation. Likewise, the fatty acid composition of phospholipids from the granules in general was similar to that reported for Ehrlich ascites cells. On the basis of the polar and neutral lipid ratio, the lipid granules of the ascites cells were calculated to possess lipid core diameters of 30–50 nm, which were 40–70 times smaller than those (up to 2μ) measured for the lipid granules of the intact cells by electron microscopy. The characterization of the lipid composition of the Ehrlich ascites lipid granules was completed by determining the molecular species composition of the diacyl, alkylacyl and alkenylacyl phosphatidylethanolamines and of the diacyl and alkylacyl phosphatidylcholines of the ascites cells. It is concluded that the alkyldiacylglycerols of the Ehrlich ascites cells occur largely in the cytoplasmic lipid granules, which appear to consist of many particles of the size and structure of very low density lipoproteins enclosed in membranous sacs.
Lipids 23, 398–404 (1988).

The composition of fatty chains of neutral lipids and phospholipids of whole Ehrlich ascites cells and of a membrane fraction has been described in great detail by Wood (1). More recent studies have confirmed and extended these findings (2–5), and the characteristic presence of excessive amounts of ether-linked phospholipids and neutral lipids in tumor tissues and neoplastic cells now is generally recognized (6,7).

Early work by DiPaolo et al. (8) had identified a lipoid granule fraction in Ehrlich ascites cells, which later workers had not bothered to isolate and examine separately. This particle had been observed to contain a high proportion of an unidentified neutral lipid, which was recovered along with the steryl esters from a silicic acid column. We recently have reisolated the granule fraction from Ehrlich ascites cells and have shown that the unknown ester is an alkyldiacylglycerol similar to that previously characterized by Wood and Snyder (9) from whole cell extracts. In the course of this study, we also have determined the molecular association of the fatty chains in the diacyl, alkylacyl and alkenylacylglycerol moieties of the glycerophospholipids of whole Ehrlich ascites cells, which had not been reported previously.

MATERIALS AND METHODS

Cells. The cells used in this study were a hyperdiploid strain of the Ehrlich-Lettre ascites carcinoma. It was obtained originally from the tissue bank of the Roswell Park Memorial Cancer Institute, Buffalo, NY. The tumor was maintained by making serial transplants of 0.2 ml ascites fluid containing cells intraperitoneally into female mice of the CFW strain, or of the Connaught strain, as obtained in this university. The procedure for maintaining the tumor was essentially as described (10). After 10 days of tumor development, the mice were killed by anesthesia using diethyl ether, and the skin of the enlarged abdomen was punctured by scissors, after swabbing the area with 70% ethanol. The fluid was quickly drained into a collecting vessel. The fluid generally was the color of serum and contained mainly suspended ascites cells. Very few erythrocytes were detected by microscopic examination. The fluid was used for further transplants as described or for the preparation of the lipid granules enclosed in these cells.

Lipid granules. For the preparation of the intracellular particles, the ascites fluid was centrifuged for 10 min at 200 × g at 4 C. The supernatant fluid was discarded, and the cells were washed at least two times with 0.9% NaCl; the centrifugation was repeated. Packed cell pellets obtained from at least 10 mice were pooled and suspended in cold distilled water in a ratio of 1:10 and the lipid particles collected essentially as described by DiPaolo et al. (8). The suspension was stirred in the cold or was gently homogenized to facilitate cell rupture. This was carried out with a motor-driven homogenizer using a Teflon and glass mortar and pestle. The stages in cell rupture and release of the particles were monitored by phase contrast microscopy. The particles and lipid-containing components of the cell could be obtained by flotation, if allowed to stand. It proved to be more convenient to adjust the suspending medium to a concentration of 0.25 M sucrose and to centrifuge for approximately 30 min at 9,000 × g. The supernatant then was free of mitochondria and cell debris, and the lipid particles in it could be collected by centrifugation at 145,000 × g for two hr using a Spinco L-65 centrifuge. The floating white band at the top of the centrifuge tube was recovered using a Pasteur pipet.

Lipid analyses. The lipids of the cells and the lipid granules were extracted with chloroform/methanol (2:1, v/v) (11) and the solvents evaporated under nitrogen. The

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Abbreviations: TMS, trimethylsilyl; VLDL, very low density lipoproteins; GLC, gas liquid chromatography; TLC, thin layer chromatography.

total lipid extracts were resolved by preparative thin layer chromatography (TLC) and the individual neutral and phospholipid classes recovered as described (12). An aliquot of the total lipid extract was applied to a 20 × 20 cm Silica Gel H plate (250 μm thick layer) about 2.5 cm from the bottom edge, and the plate was developed with a polar solvent system made up of chloroform/methanol/4 N ammonia (65:25:4, v/v/v), which resolved phosphatidylcholine and phosphatidylethanolamine from the sphingomyelin and phosphatidylserine and phosphatidylinositol, which overlapped. The total neutral lipid fraction was carried to the solvent front. The lipid bands were located by dichlorofluorescein and the gel scrapings were eluted with chloroform/methanol/acetic acid/water (50:39:1:10, v/v/v/v) (13). The recovered neutral lipids were resolved into free sterols, free fatty acids, triacylglycerols, alkyldiacylglycerols and cholesteryl esters using petroleum ether (bp 30–60 C)/diethyl ether (60:10, v/v) as the developing solvent (14). The bands were located with fluorescein and were extracted with chloroform/methanol (2:1, v/v). The individual phospholipid classes were digested with phospholipase C from *Bacillus cereus* as described (15) and the diradyl moieties recovered by extraction with diethyl ether. An aliquot of the diradylglycerol fraction was converted into the trimethylsilyl (TMS) ethers for direct gas liquid chromatographic (GLC) examination (15). The remaining diradylglycerols were converted into the t-butyldimethylsilyl (t-BDMS) ethers and resolved by TLC into the diacyl, alkylacyl and alkenylacyl subclasses using benzene as the developing solvent as described (16).

GLC of the TMS and t-BDMS ethers of the diradylglycerols was performed (15,16) on a Hewlett-Packard model 5880 automatic gas chromatograph with a level IV terminal and 10 m open tubular glass column (0.25 mm i.d.) with its wall coated with SP-2330 liquid phase (68% cyanopropyl and 32% phenylsiloxane) supplied by Supelco (Bellefonte, PA). The temperatures of the injector and detector were both maintained at 270 C. Separations of diradylglycerol derivatives were made isothermally at 250 C using a split injection (split ratio 7:1) and hydrogen as a carrier gas. Separations of fatty acid methyl esters and dimethylacetals were made by temperature programming the column from 100 to 160 C at 20 C/min and then at 2 C/min or alternatively from 100 to 180 C at 20 C/min and then at 2 C/min, using hydrogen as carrier gas. The peaks were identified by reference to previous work with well-characterized $AgNO_3$-TLC fractions of the t-BDMS ethers derived from rat heart phosphatidylethanolamines (16). Carbon number profiles of the intact cholesteryl esters, triacylglycerols and alkyldiacylglycerols isolated by TLC, and the lipid profiles of the total lipid extracts of the ascites cells and of the lipid granules were determined by GLC on 50 cm × 0.2 cm i.d. stainless-steel columns packed with 3% OV-1 (a methylsilicone polymer) on 100–120 mesh Gas-Chrom (Applied Science Laboratories, State College, PA) using nitrogen as the carrier gas in the temperature range 175–355 C, as described (17). The peak identification and composition of samples were calculated in relation to tridecanoylglycerol internal standard. Fatty acid methyl esters and dimethyl acetals were prepared from the neutral and phospholipids by methylation with 6% H_2SO_4 in methanol, while methyl esters were prepared by methylation with sodium methoxide. The alkylglycerol moieties of the glycerolipids were determined by GLC on the short nonpolar columns (17) following saponification and trimethylsilylation.

Microscopy and histochemistry. The Ehrlich-Lettre ascites carcinoma cells and isolated lipid granules (Morrish, R. B., and Kay, E.R.M., unpublished observations) were examined by phase contrast microscopy. Oil red 0 was used as stain, if required. In some cases, the cells and isolated lipid granules were examined by electron microscopy. For this purpose, the cells and the granules were fixed in phosphate buffered (pH 7.4) 5% glutaraldehyde, followed by post-fixation in 2% osmium tetroxide (pH 7.4).

RESULTS

Physical properties of cells and granules. Figure 1 shows a preparation of ascites cells fixed and stained with glutaraldehyde and osmic acid. The cells are roughly spherical in shape, with numerous microvilli on the surface. The cytoplasm contains several osmic acid dense structures, corresponding in size (less than 2μ), number and distribution, with the refractile bodies seen in the phase contrast microscope. The particles seen within the cells by electron microscopy are generally of fairly uniform size. When the flotation and washing procedures are carried out with care, the isolated lipid particles appear clean and similar in size to those seen intracellularly and only occasionally contaminated with ribosomal elements. Likewise, phase contrast microscopy and the

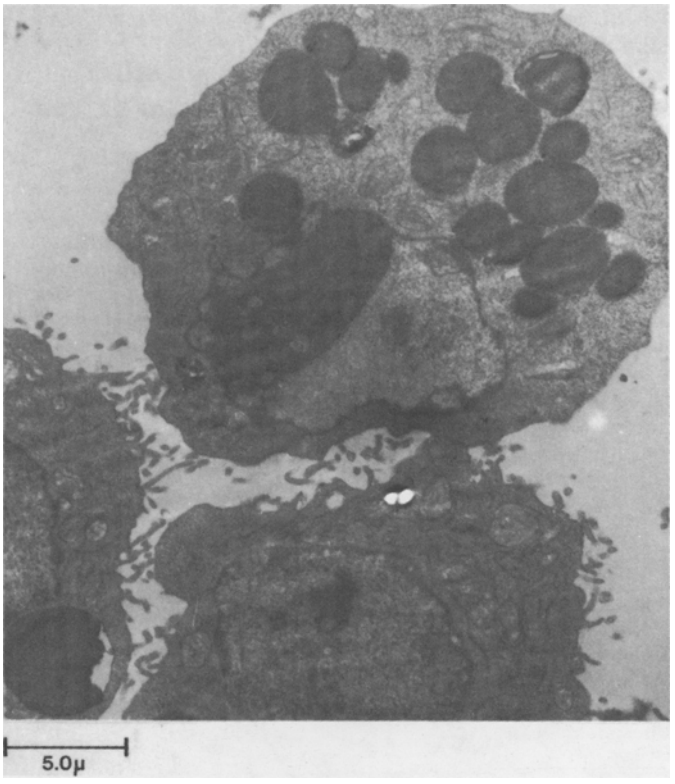

FIG. 1. Transmission electron micrograph of ascites cells fixed in buffered 5% glutaraldehyde (phosphate buffer, pH 7.4), followed by post-fixation in 2% osmium tetroxide (pH 7.4). Magnification 15,000 times.

application of oil red 0 staining revealed uniform particles free of extreneous material.

Chemical composition of lipid granules. Table 1 gives the lipid class composition of the lipid granules obtained in the present work and by DiPaolo et al. (8) along with the values for whole ascites cells tabulated by Wood (1). About 50% of the lipid in the granules is triacylglycerol, and about 30% is alkyldiacylglycerol. Cholesteryl esters and free cholesterol each make up about 3% of the total, while phospholipids account for about 10% of the particle lipid. These values are close to those reported by DiPaolo et al. (8) and differ significantly from those reported for whole ascites cells (9) and a cell membrane fraction (1,18), which contain much smaller proportions of triacylglycerols and especially alkyldiacylglycerols. The isolated particles gave neutral/polar lipid ratios corresponding to calculated (19) lipid core average diameters of 30–50 nm.

Table 2 compares the carbon number distribution of the cholesteryl ester, alkyldiacylglycerol and triacylglycerol fractions of the lipid granules as determined in the present work along with the corresponding values from whole ascites cells and the membrane fraction analyzed previously (1,18). The overall range and proportion of the carbon numbers of the triacylglycerols and alkyldiacylglycerols are similar to those of the corresponding neutral lipids of whole Ehrlich ascites cells. There is a much higher proportion of the longer chain length components in the cholesteryl esters of the lipid granules than in the membranes or in whole cells analyzed previously (1,18). Cholesterol was the only sterol present. The results obtained by analysis of intact lipid molecules are supported by analysis of the component glyceryl ethers and fatty acids.

Table 3 compares the alkylglycerol composition of the alkyldiacylglycerol fraction of the lipoid granules determined in this study and those recovered from the alkyldiacylglycerol fraction of whole Ehrlich ascites cells (1). There are significant differences in the actual fatty chain composition, but the carbon number proportions do not differ too much, thus accounting for the similarity in the overall carbon number distribution of the alkyldiacylglycerols from the two sources.

Table 4 compares the fatty acid composition of the triacylglycerol and alkyldiacylglycerol fractions isolated from the fat granules by us and by DiPaolo et al. (8) and of the choline and ethanolmine phosphatides isolated from the fat granules and the whole cells in our laboratory. While the fatty acids of the triacylglycerol fractions from both isolations possess closely similar compositions, the alkyldiacylglycerol fraction isolated by DiPaolo et al. (8) contains much more oleic and less docosahexaenoic acid

TABLE 1

Lipid Class Composition of Ehrlich Ascites Fat Granules

Lipid fraction	Lipid granules		Cells
	Present study*	Ref. 8	Ref. 1
	Weight %		
Cholesteryl esters	3	3	9–12
Alkyldiacylglycerols	30	35**	1–2
Triacylglycerols	51	42	14–18
Free cholesterol	3	3	8–11
Phospholipids	10	11	57–70
Other	4	6	0–1

*As obtained from quantitative GLC analyses of neutral and phospholipid fractions prepared by TLC and from GLC profiles of total lipids.

**Liebermann-Burchard negative material isolated by adsorption chromatography along with cholesteryl esters.

TABLE 2

Carbon Number Distribution in Cholesteryl Ester, Alkyldiacylglycerol and Triacylglycerol Fractions of the Fat Granules, Membranes and Whole Ehrlich Ascites Tumor Cells

Lipid components	Fat granules (Present study)	Membranes (Ref. 18)	Ehrlich ascites (Ref. 1)
		Weight %	
Cholesteryl esters			
41	3.1	2	2
43	6.3	22	11
45	39.0	59	59
47	27.9	10	19
49	17.1	1	8
51	4.4		trace
53	2.2		—
Alkyldiacylglycerols			
46	2.6		0.8
48	4.0		6.3
50	10.0		18.2
52	17.8		24.4
54	21.0		20.1
56	20.6		17.6
58	14.8		9.2
60	5.3		2.5
62	3.8		1.1
Triacylglycerols			
46	1.9		0.4
48	2.5		1.3
50	6.2		6.9
52	14.6		21.7
54	26.3		32.4
56	21.9		19.1
58	17.3		13.2
60	7.1		3.8
62	2.1		1.1

TABLE 3

Composition of Alkylglycerol Moieties of the Alkyldiacylglycerols of the Fat Granules of Ehrlich Ascites Cells*

Fatty chain	Lipid granules (Present study)	Cells (Ref. 1)
14:0	0.3	0.5
16:0	35.3	45.0
16:1	8.5	0.0
18:0	17.7	28
18:1	36.6	24
20:0	1.6	1

*The alkylglycerol moieties of the alkyldiacylglycerols were identified and quantitated by GLC on the short nonpolar columns (17) following saponification and trimethylsilylation.

TABLE 4

Fatty Acid Composition of Neutral Lipid and Phospholipid Classes of Fat Granules and Whole Ehrlich Ascites Cells

Fatty acid	Lipid classes							
	GEDE		TG		PC		PE	
	FG	Ref. 8	FG	Ref. 8	FG	Cells*	FG	Cells*
	Mol %							
8:0		1.9		3.2				
14:0	tr	4.1	2	1.6	1	0.9	1	0.7
16:0	16	8.5	13	18.6	13	21.6	8	11.6
16:1ω7	5	4.3	2	2.0	5	2.1	1	0.8
18:0	8	3.0	14	12.5	18	16.7	19	23.2
18:1ω9+ω7	19	49.1	26	29.2	19	16.3	18	14.6
18:2ω6	16	18.3	23	27.2	18	22.1	21	18.4
18:3ω3	3		3	1.2	7		1	
20:3ω6	2		1		3	1.4	2	1.1
20:4ω6	9	9.9	4	2.3	9	7.5	14	12.4
20:5ω3						0.2		0.7
22:4ω6						1.2		1.1
22:5ω3						1.0		2.4
22:6ω3	19		7		6	4.3	14	12.5

*The choline phosphatides of whole cells also contained 0.9% 16:0 and 0.4% 18:0 alkenyl, and a total of 16.5% alkyl chains, while the ethanolamine phosphatides also contained 7.1% 16:0, 2.8% 18:0 and 1.7% 18:1 alkenyl as well as a total of 6.5% alkyl chains.

Cells, whole Ehrlich ascites cells; FG, fat granules; GEDE, glyceryl-etherdiesters (alkyldiacylglycerols); PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerols. Fatty acids are identified by their carbon number:double bond number. The ω-values indicate the location of the last double bond by the number of carbons from the methyl terminal.

than that obtained in this study. Although the longer chain acids could have been incompletely recovered in the earlier work, there is no obvious explanation for the high proportion of oleic relative to linoleic acid in this fraction. The fatty acid composition of the choline and ethanolamine phosphatides of the lipid granules is closely similar to that of the corresponding lipid classes from whole cells, and not unlike that described by Wood et al. (18) for the ascites cell membranes. However, the choline phosphatides of whole cells contained about 15% alkyl chains and the ethanolamine phosphatides 6% alkyl and 13% alkenyl chains, which were not determined in the phosphatide fractions of the lipid granules.

Molecular species of glycerolipids in intact cells. Figure 2 gives the polar capillary GLC profiles of the total, diacyl, alkenylacyl and alkylacylglycerol subclasses of the ethanolamine phosphatides of whole tumor cells. The preliminary separation of the various subclasses of phosphatides has permitted effective resolution of the individual molecular species, which overlap extensively in the chromatogram recorded for the mixed ethanolamine phosphatides. In this instance, the alkenylacyl and alkylacylglycerol species possessed by far the largest proportion of the longer polyunsaturated chain lengths, reflecting mainly the presence of a high proportion of docosahexaenoic acid. A similar observation has been made by Nakagawa and Waku (20).

Figure 3 shows the polar capillary GLC profiles of the diacyl and alkylacyl subclasses of the phosphatidylcholines of Ehrlich ascites tumor cells. There is an essentially complete resolution of all molecular species, although some minor ones do not project very clearly above the baseline. The combinations of the major chain lengths are markedly different in the two types of the phosphatidylcholines, with the alkylacyl species containing a significantly higher proportion of the longer polyunsaturated chain lengths.

Table 5 lists the various molecular species identified by polar capillary GLC along with the quantitative estimates for each subclass of the two phosphatides in whole Ehrlich ascites cells. The diacyl and the alkylacylglycerol subclasses of the choline phosphatides made up, respectively, 70% and 26% of the total. The remaining 4% was contributed by alkenylacyl species, which were not analyzed in detail for this phospholipid class. Wood and Snyder (9) reported 64% and 36%, respectively, for the diacyl and alkylacylglycerol subclasses of phosphatidylcholine from whole ascites cells. The diacyl, alkylacyl and alkenylacylglycerol subclasses of the ethanolamine phosphatide made up, respectively, 61%, 13% and 26% of total. Wood and Snyder (9) reported 56%, 29% and 15% of the total in the corresponding subclasses of the ethanolamine phosphatide of whole ascites cells, which, however, is the reverse of our values for the alkylacyl and alkenylacyl species. Summation of the major molecular species by carbon number gave molecular weight distributions for the diacyl, alkylacyl and alkenylacylglycerols of these phosphatides, which were rather similar to those obtained by Wood and Snyder (9) by GLC on nonpolar columns. Direct analyses of the fatty chain pairing in individual molecular species of the glycerophospholipids of Ehrlich ascites cells had not been reported.

DISCUSSION

The present study confirms the earlier reported (8) presence of lipid granules in Ehrlich ascites tumor cells. Isolation and chemical analysis of the particles gave calculated lipid core diameters, which were 40 to 70 times smaller than the diameters of the lipid granules seen in the intact Ehrlich ascites cells. Because the calculation (19) represents a valid method of estimating the particle size of lipoproteins, it is suggested that each granule in the cell is a membranous bag filled with lipid particles of a size and structure comparable to very low density lipoproteins (VLDL). It was not determined if there are any apolipoproteins associated with the fat granules of the Ehrlich ascites cells.

The previously unidentified fatty ester, which on silicic acid columns had migrated with the cholesteryl esters, was identified as a mixture of alkyldiacylglycerols, which Wood and Snyder (9) had characterized from whole Ehrlich ascites cells. However, the relative proportion of alkyldiacylglycerols in the fat granules was many times higher than that in the whole cells and approached the quantitates estimated for the Liebermann-Burchard negative material isolated along with the cholesteryl ester fraction by DiPaolo et al. (8). We could not confirm the high proportion of oleic acid reported for the alkyldiacylglycerol fraction of the fat granules by DiPaolo et al. (8). The small cholesteryl ester fraction of the lipid granules was found to possess an unusually high proportion of the longer chain length polyunsaturated fatty acids (C_{20} and

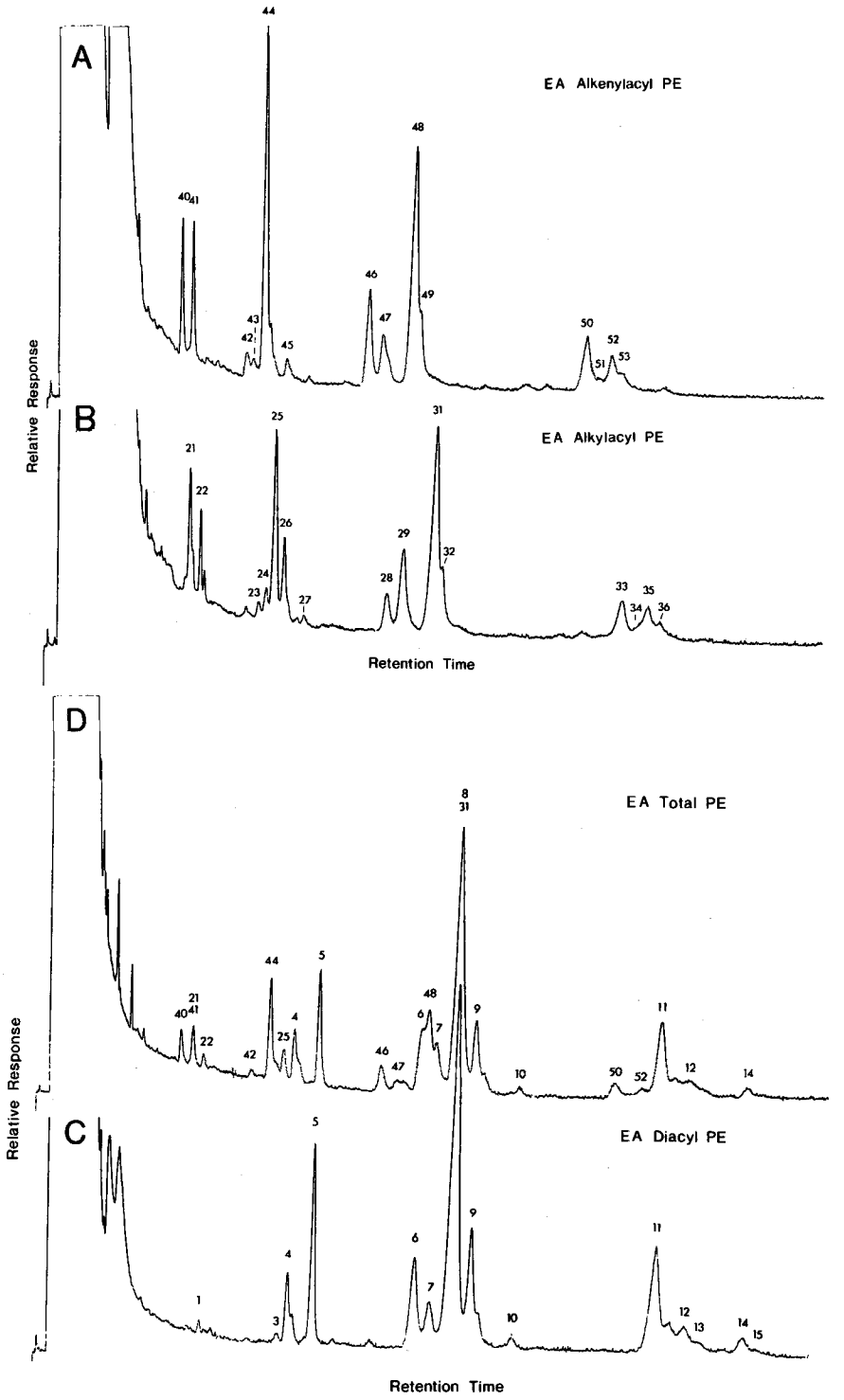

FIG. 2. GLC resolution of the t-BDMS ethers of the diradylglycerol moieties of the ethanolamine phosphatides isolated from the Ehrlich ascites cells of mice. A, alkenylacyl; B, alkylacyl; C, diacyl; D, total. Peak identification: 1, 16:0 16:0; 2, 16:0 16:1ω9 + 16:0 16:1ω7; 3, 16:0 18:0; 4, 16:0 18:1ω9 + 16:0 18:1ω7; 5, 16:0 18:2; 6, 18:0 18:1ω9; 7, 18:1ω9 18:1ω9; 8, 18:0 18:2 + 16:0 20:4; 9, 18:1ω9 18:2 + 18:1ω7 18:2; 10, 18:2 18:2 + 16:0 20:5; 11, 18:0 20:4; 12, 18:1ω9 20:4; 13, 18:1ω7 20:4; 14, 16:0 22:6; 15, 16:0 22:5; 16, 18:0 22:6; 20, 16:0′ 16:0; 21, 16:0′ 18:1ω9; 22, 16:0′ 18:2; 23, 18:0′ 18:1ω9; 24, 18:1′ 18:1ω9; 25, 18:0′ 18:2 + 16:0′ 20:4; 26, 18:1′ 18:2; 27, 16:0′ 20:5; 28, 18:0′ 20:4; 29, 18:1′ 20:4 + 16:0′ 22:4; 30, UNKN; 31, 16:0′ 22:6; 32, 16:0′ 22:5; 33, 18:0′ 22:6; 34, 18:0′ 22:5; 35, 18:1′ 22:6; 36, 18:1′ 22:6 + 18:1′ 22:5; 40, 16:0″ 18:1ω9; 41, 16:0″ 18:2; 42, 18:0″ 18:1ω9; 43, 18:1″ 18:1ω9; 44, 16:0″ 20:4 + 18:0″ 18:2; 45, 16:0″ 20:5; 46, 18:0″ 20:4; 47, 18:1″ω9 20:4 + 18:1″ω7 20:4; 48, 16:0″ 22:6; 49, 16:0″ 22:5; 50, 18:0″ 22:6; 51, 18:0″ 22:5; 52, 18:1″ 22:6; 53, 18.1″ 22:6 + 18:1″ 22:5. Peak 11 eluted at 23.22 min.; H_2 carrier gas, 6 psi. Other GLC conditions as given in text. (′) and (″) designate alkyl and alkenyl fatty chains, respectively.

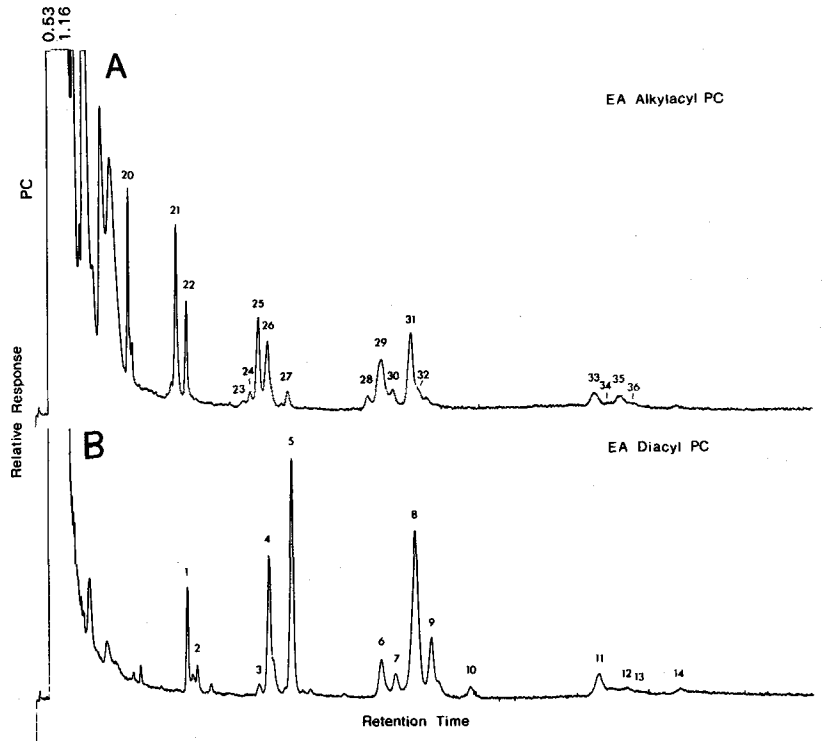

FIG. 3. GLC resolution of the t-BDMS ethers of the diradylglycerol moieties of the choline phosphatides isolated from the Ehrlich ascites cells of mice. A, alkylacyl; B, diacyl. Peak identifications and GLC conditions as given in Figure 2.

TABLE 5

Molecular Species of Diradylglycerol Moieties of Phosphatidylethanolamine and Phosphatidylcholine of Ehrlich Ascites Cells

Molecular species	Phosphatidylethanolamine			Phosphatidylcholine	
	Diacyl	Alkyl acyl	Alkenyl acyl	Diacyl	Alkyl acyl
			Mol %		
16:0 16:0	0.5	0.7	—	5.5	7.8
16:0 16:1	—	—	—	2.3	1.5
16:0 18:0	0.5	—	—	0.8	1.1
16:0 18:1	4.4	6.4	4.2	13.5	12.0
16:0 18:2	9.1	3.1	5.0	17.6	6.7
18:0 18:1	7.4	1.2	1.4	7.5	0.9
18:1 18:1	4.0	1.9	1.3	5.1	1.6
18:0 18:2	29.3	1.6	2.3	23.1	0.7
16:0 20:4	5.3	10.9	19.9	4.6	8.2
18:1 18:2	10.7	6.2	trace	8.8	9.6
18:2 18:2 + 16:0 20:5	1.3	0.9	2.3	1.4	—
18:0 20:4	11.3	4.0	8.0	5.1	2.5
18:1 20:4 + 16:0 22:4	4.7	9.5	6.2	1.6	11.2
16:0 22:5	0.6	5.8	5.6	0.4	2.0
16:0 22:6	1.9	23.6	21.6	0.4	14.3
18:0 22:5	0.8	1.5	1.3	—	1.4
18:0 22:6	3.5	6.0	6.1	0.7	2.0
18:1ω9 22:6	—	4.4	3.7	—	3.2
18:1ω7 22:6 + 18:1 22:5	—	2.5	2.9	—	1.9
Other	4.7	9.8	8.2	1.6	11.4
% PL Class	61	13	26	70	26

Fatty chain identification by number of carbons:number of double bonds. The fatty acids on the left side of the molecular species pair is to be read as either acyl, alkyl or alkenyl chain depending on the phosphatide subclass.

C_{22}), when compared with the cholesteryl esters of the total Ehrlich ascites cell.

An examination of the phospholipid composition of the lipid particles was limited to a determination of the fatty acids, which was rather similar to that found for the corresponding phospholipid classes of the ascites cell. However, the phosphatides isolated from whole cells also contained alkyl and alkenyl fatty chains, which would not have been detected in the glycerophosphatides isolated from the fat granules, because the sodium methoxide used for transmethylation would not break the ether linkages. Although Wood et al. (1,18) have claimed an essential absence of both alkyl and alkenylacylglycerol subclasses from the choline and ethanolamine phosphatides of a membranous material isolated from Ehrlich ascites cells, it is unlikely that the alkyl and alkenyl ether phosphatides would be absent from the fat granules, which presumably depend upon microsomal lipid synthesis (4,21). Furthermore, Friedberg and Halpert (3) have reported that the amounts of *O*-alkyl lipids in the surface membranes of Ehrlich ascites cells are the same or greater than the amounts of *O*-alkyl lipid found in the microsomes, mitochondria and whole cell homogenates. These workers could not account for the findings of Wood et al. (18). In view of these considerations, it is likely that the molecular species composition of the choline and ethanolamine phosphatides of the whole cells also represents that of the fat granules. However, a possible preferential concentration of the alkyl and alkenyl species of the phosphatides in the granules could not be ruled out in view of the marked enrichment in the alkyldiacylglycerols there. An extensive equilibration of cholesteryl esters and the alkyldiacyl and triacylglycerols between their sites of synthesis in the cell and the lipid granules also would be unlikely in view of the marked differences in the qualitative and quantitative composition of the molecular species.

There has been little speculation about the origin and the metabolic role of the fat granules in the Ehrlich ascites tumor cells. A slow staining of these granules with Janus Green B and their location in opposition to mitochondria have led MacKenzie et al. (21) to suggest that they may possess enzymes and furnish substrate for mitochondria. Because no specific enzymic activities were demonstrated, it is possible that the staining was due to an association of apoproteins with these particles, which would be anticipated in view of the hepatic origin of the ascites cells and of their general similarity to VLDL. Alkyldiacylglycerols have been observed to accumulate also in other cancer cells (6), in Ehrlich ascites cells (22) and in normal cells in culture (23). This has been attributed to an enhanced rate of glucose utilization via the dihydroxyacetone pathway (6) combined with a decreased activity of the alkyl ether cleavage enzyme (24). It also has been suggested (1) that the ascites cells may have reverted to a more primitive stage of lipid metabolism characterized by the ability of direct utilization of products of lipase activity. The presence of specialized lipid granules containing the alkyldiacylglycerols, however, has not been demonstrated in tumor cells other than Ehrlich ascites cells. Neither have attempts been made to isolate them from any other cell types.

This study provides the first quantitative analysis of the specific molecular association of the fatty chains in the choline and ethanolamine phosphatides of the Ehrlich ascites tumor cells, although extensive investigations have been reported of the carbon number distribution (1) and of fatty acid composition of the subfractions of the diacyl, alkylacyl and alkenylacylglycerol moieties of these phospholipids following $AgNO_3$-TLC (4,5). The complete resolution of the molecular species of the diradylglycerols obtained on the polar capillary columns confirms the preferential association of the long chain polyunsaturated fatty acids with the alkyl and alkenyl chains in the ethanolamine phosphatides. Likewise, there is a good agreement between the present direct and previous indirect analyses of the other major molecular species of diacyl and alkylacyl subclasses of the choline and ethanolamine phosphatides. A further rationalization of the composition of the molecular species of the neutral and polar glycerolipids of the Ehrlich ascites tumor cells and of the lipid granules isolated from them requires a better understanding of glycerolipid metabolism in general.

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REFERENCES

1. Wood, R. (1973) in *Tumor Lipids: Biochemistry and Metabolism* (Wood, R., ed.), pp. 139-182, American Oil Chemists' Society, Champaign, IL.
2. Brenneman, D.E., McGee, R., Liepkalns, V.A., and Spector, H.A. (1975) *Biochim. Biophys. Acta 388,* 301-304.
3. Friedberg, S.J., and Halpert, M. (1978) *J. Lipid Res. 19,* 57-64.
4. Waku, K., and Nakazawa, Y. (1978) *Eur. J. Biochem. 88,* 489-494.
5. Masuzawa, Y., Nakazawa, Y., Waku, K., and Lands, W.E.M. (1982) *Biochim. Biophys. Acta 713,* 185-192.
6. Scott, C.C., Heckman, C.A., and Snyder, F. (1979) *Biochim. Biophys. Acta 575,* 215-224.
7. Spener, F. (1983) in *Ether Lipids: Biochemical and Biomedical Aspects* (Mangold, H.K., and Paltauf, F., eds.) pp. 239-259, Academic Press, Inc., New York, NY.
8. DiPaolo, J.A., Hening, A., and Carruthers, C. (1963) *Proc. Soc. Exp. Biol. Med. 13,* 68-70.
9. Wood, R., and Snyder, F. (1969) *Arch. Biochem. Biophys. 131,* 478-494.
10. Kay, E.R.M. (1965) *Cancer Res. 25,* 764-768.
11. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
12. Skipski, V.P., Peterson, R.F., and Barclay, M. (1964) *Biochem. J. 90,* 374-378.
13. Arvidson, G.A.E. (1968) *Eur. J. Biochem. 4,* 478-486.
14. Myher, J.J., Kuksis, A., Breckenridge, W.C., McGuire, V., and Little, J.A. (1985) *Lipids 20,* 90-101.
15. Myher, J.J., and Kuksis, A. (1982) *Can. J. Biochem. 60,* 638-650.
16. Myher, J.J., and Kuksis, A. (1984) *Can. J. Biochem. Cell Biol. 62,* 352-362.
17. Kuksis, A., Myher, J.J., Marai, L., and Geher, K. (1975) *J. Chromatogr. Sci. 13,* 423-430.
18. Wood, R., Anderson, N.G., and Swartzendruber, D.C. (1970) *Arch. Biochem. Biophys. 141,* 190-197.
19. Kuksis, A., Breckenridge, W.C., Myher, J.J., and Kakis, G. (1978) *Can. J. Biochem. 56,* 630-639.
20. Nakagawa, Y., and Waku, K. (1985) *Eur. J. Biochem. 152,* 569-572.
21. Mackenzie, C.G., Mackenzie, J.B. and Reiss, O.K. (1967) *J. Lipid Res. 8,* 642-645.
22. Cheng, S., Piantodosi, C., and Snyder, F. (1967) *Lipids 2,* 193-194.
23. Scott, C.C., Heckman, C.A., Nettesheim, P., and Snyder, F. (1979) *Cancer Res. 39,* 207-214.
24. Soodsma, J.F., Piantodosi, C., and Snyder, F. (1970) *Cancer Res. 30,* 309-311.

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Effect of Dietary Vitamin E and Selenium on Susceptibility of Brain Regions to Lipid Peroxidation

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The effect of dietary vitamin E and/or selenium (Se) supplementation (200 IU and/or 0.2 ppm, respectively) or deficiency for two months on lipid peroxidation in cerebrum, cerebellum, mid-brain, and brain stem of one-month-old male F344 rats was investigated. Dietary treatment had a minimal effect on weight gain of rats for the period tested. Plasma α-tocopherol (α-T) concentration and glutathione peroxidase (GSH-Px) activity were reflective of dietary treatments. Supplementation of diets with vitamin E and/or Se increased plasma α-T and/or GSH-Px activity, while diets devoid of these nutrients reduced them significantly. Increased GSH-Px activity in Se-supplemented rats was further enhanced by vitamin E supplementation. Differential concentrations of α-T among brain regions were affected by dietary vitamin E but not by Se. In vitro lipid peroxidation of brain homogenates was inhibited by dietary vitamin E supplementation and increased by deficiency. Addition of 0.25 mM ascorbic acid or 0.1 mM of Fe^{2+} to brain homogenates markedly increased in vitro lipid peroxidation. Ascorbic acid-induced lipid peroxidation was inversely correlated with dietary vitamin E and Se in cerebrum. In vitro Fe^{2+}-addition induced the greatest stimulation of lipid peroxidation, with cerebellum and brain stem of vitamin E-deficient rats showing the highest response to Fe^{2+} challenge. These findings indicate that concentrations of α-T among the brain regions are different and can be altered by dietary vitamin E treatments, cerebellum and brain stem are more susceptible to in vitro challenge by peroxidative agents than other regions, and the degree of lipid peroxidation of brain regions is partially affected by dietary vitamin E but not by Se in the levels tested. *Lipids 23,* **405–409 (1988).**

Vitamin E deficiency in experimental animals induces pathological changes in the reproductive, cardiovascular and nervous systems, and in other tissues (1–4). Neurological signs of chronic vitamin E deficiency include axonal swelling and dystrophy, demyelination, increased oxidative enzyme activity and neuronal pigment accumulation (1,4–8). Neuropathological observations linking vitamin E deficiency to neurological disorders in humans indicate the importance of dietary vitamin E in central nervous system function (9–11). The interaction between dietary vitamin E and selenium (Se) incorporated into glutathione peroxidase (GSH-Px) has been well-documented (12,13). The concentration and uptake of vitamin E and the activity of Se-dependent GSH-Px vary among the brain regions (14–17). The antioxidative functions of vitamin E and Se suggest that changes in the concentration of these nutrients in particular brain regions may influence regional susceptibility to lipid peroxidation. Most information on the influence of antioxidants on brain lipid peroxidation has emerged from use of either whole brain or cerebrum tissue (18–21). In a previous study, we reported that lipid peroxidation of cerebrum is affected by dietary vitamin E and Se (22). Different brain regions in rats are well-defined and composed of a variety of neuronal processes with specific biochemical and neurological functions. Therefore, this study was undertaken to extend our previous work to examine the influence of feeding vitamin E- and Se-deficient and/or supplemented diets on the lipid peroxidative capacity of different brain regions of rat: cerebrum, cerebellum, mid-brain and brain stem. Our findings are consistent with the concept that vitamin E protects lipid components of neuronal tissue from free radical reactions and peroxidation.

MATERIALS AND METHODS

Forty-eight, one-month-old male Fischer 344 rats (Charles River Breeding Lab., Inc., Wilmington, MA) were randomly assigned to six dietary treatment groups. Group A (control) received the basal diet (containing no vitamin E and Se, Table 1) supplemented with 30 IU α-tocopheryl acetate/Kg diet and 0.1 ppm Se as sodium selenite as recommended by National Research Council and National Academy of Science for normal growth and reproduction (23). Group B (+E+Se) received the basal diet supplemented with 200 IU α-tocopheryl acetate and 0.2 ppm Se. Group C (−E+Se) received the basal diet supplemented with 0.2 ppm Se. Group D (+E−Se) received the basal diet supplemented with 200 IU α-tocopheryl

TABLE 1

Composition of Basal Diet

Ingredient	Percent
Torula yeast[a]	30.0
Sucrose[a]	56.7
α-Tocopherol stripped lard[a]	5.0
α-Tocopherol stripped corn oil[a]	2.0
DL-Methionine[a]	0.3
Mineral mix (selenium free)[a,b]	5.0
Vitamin mix (vitamin E free)[c]	1.0
Total	100.0

[a]Teklad, Madison, WI.

[b]Hubbell-Mendel-Wakeman (3.08 g of $MnSO_4.H_2O$ per kg of mineral mix was added). Mineral contribution from torula yeast gave final percentage concentration in diet as follows: Ca, 1.16; P, 0.77; K, 1.2: Na, 0.16; Mg, 0.10; Fe, 0.017; Cu, 0.0023; Mn, 0.0055; Zn, 0.0037.

[c]Vitamin content in 1 kg of diet: thiamine HCl, 4.0 mg; riboflavin, 4.8 mg; pyridoxine HCL, 2.0 mg; calcium pantothenate, 20.0 mg; niacin, 100.0 mg; menadione, 1.0 mg; folic acid, 2.0 mg; biotin, 1.0 mg; vitamin B_{12} (0.1% trituration), 10.0 mg; retintyl palmitate, 100 mg; cholecalciferol, 4 mg; sucrose was added to make total vitamin mix 10 g.

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Abbreviations: α-T, alpha tocopherol; MDA, malonaldehyde; TBARS, thiobarbituric acid reactive substances.

acetate/Kg diet. Group E (−E−Se) received only the basal diet. Group F was pair fed to the double-deficient group E with the group A diet.

Rats were individually housed in stainless steel wire mesh cages and maintained at 22 C with 12 hr light-dark cycles. All the animals were fed ad libitum except the pair-fed rats (group F). After eight wk of dietary treatment, animals were fasted overnight then killed by decapitation. Blood was collected in heparinized tubes and plasma separated and stored at −70 C. Brain was removed from the skull, placed on an ice-cold platform, and immersed with ice-cold buffer. The cerebrum, cerebellum, mid-brain (including hypothalamus, striatum, and hippocampus) and brain stem (including medulla oblongata and pons) were dissected with precooled dissecting tools within one to two min of decapitation. Ten percent homogenates of the brain regions were prepared using ice-cold 1.15% KCl. Following addition of α-tocopheryl acetate containing 0.1% butylated hydroxytoulene in ethanol to the homogenate or plasma, α-tocopherol (α-T) was extracted with hexane, dried under a stream of nitrogen and reconstituted in ethanol. α-T was measured by the modified high performance liquid chromatography method described by Bieri et al. (24) using 100% methanol as the mobile phase and fluorometric detection (Exc:292, Emm:340 nm) for enhanced sensitivity. Detection limits for α-T in plasma and brain samples were 0.2 μg/ml and 10 μg/mg, respectively. Glutathione peroxidase (GSH-Px) activity in plasma was measured by the method of Paglia and Valentine (25). Thiobarbituric acid reactive substances (TBARS) were measured (26) as an index of lipid peroxidation in brain homogenates with and without incubation at 37 C for two hr, and in the presence and absence of 0.25 mM L-ascorbic acid or 0.1 mM Fe^{2+} as ferrous sulfate. Malonaldehyde bisdimethylacetal was used as a standard to calculate the molar equivilance of malonaldehyde (MDA) for TBARS assessment (27). Total protein in brain homogenates was measured by the Lowry method (28). Factorial analysis of variance and Duncan multiple means range test at 5% probability were employed for statistical analysis of the data.

RESULTS

Overall weight gain of animals among the groups was not significantly affected by dietary treatments for the period tested. The highest gains were observed in rats fed the vitamin E-supplemented diets (431% for group B and 451% for group D), while the lowest weight gains were noted in rats fed double-deficient diet (group E) and the matched pair-fed control (group F) (345% and 337%, respectively).

Plasma GSH-Px activity and α-T concentration in plasma and brain regions are shown in Table 2. GSH-Px activity and α-T concentration of plasma reflected dietary treatments. Rats fed diets supplemented with either vitamin E or Se showed increased plasma α-T or GSH-Px activity, while those fed diets devoid of these nutrients had lower plasma α-T or GSH-Px activity, respectively. Presence of vitamin E in Se-supplemented diets appeared to enhance the increment of plasma GSH-Px activity (group B vs group C). The decrease of plasma GSH-Px in the rats fed Se-deficient diets (group D vs group E) was not alleviated by vitamin E supplementation. Se deficiency or supplementation had no effect on plasma α-T concentrations from rats fed vitamin E supplemented or deficient diets (group D vs group B and group E vs group C). Plasma α-T and GSH-Px activity of pair-fed rats did not differ from those fed control diet ad libitum (group A). Concentration of α-T in brain regions varied within and between the dietary groups. The most significant increases of α-T were found in the brain regions of rats fed either +E+Se or +E−Se diets. Among the brain regions in rats fed these diets, cerebrum and mid-brain had higher α-T concentrations than cerebellum and brain stem. Conversely, feeding vitamin E-deficient diets (−E−Se and −E+Se) significantly reduced α-T in all four brain regions. Among the brain regions of rats fed these deficient diets, cerebellum and brain stem had lower α-T concentrations than cerebrum and mid-brain. The concentration of α-T in all brain regions of group F (pair-fed to group E with the control diet) was significantly higher than those of group E (−E−Se). However, cerebrum and brain stem of rats from group F had significantly lower α-T concentrations than those from group A (fed control diet ad libitum).

The influence of dietary treatment on lipid peroxidation in brain was not evident from TBARS measurements in homogenates without incubation. Endogenous TBARS levels from the brain regions of rats did not vary significantly among different dietary treatment groups (Table 3). The overall rank order of endogenous TBARS

TABLE 2

Activity of Glutathione Peroxidase in Plasma and Concentration of α-T in Plasma and Brain Regions

Dietary group	Plasma GSH-Px (U/ml)	Plasma α-T (μg/ml)	Cerebrum α-T (μg/g)	Mid-brain α-T (μg/g)	Cerebellum α-T (μg/g)	Brain stem α-T (μg/g)
A (Control)	$12.04 \pm 0.71^{a,b}$ (5)	4.57 ± 0.45^{a} (5)	14.9 ± 0.6^{a} (5)	14.7 ± 0.7^{a} (5)	$6.0 \pm 0.5^{a,c}$ (5)	10.0 ± 0.4^{a} (5)
B (+E +Se)	13.04 ± 0.24^{a} (5)	7.22 ± 0.44^{b} (8)	20.3 ± 0.8^{b} (4)	13.8 ± 1.4^{a} (4)	10.6 ± 1.9^{b} (5)	13.4 ± 0.2^{b} (4)
C (−E +Se)	10.80 ± 0.37^{b} (5)	0.24 ± 0.08^{c} (8)	6.5 ± 0.8^{c} (5)	6.2 ± 0.3^{b} (5)	2.7 ± 0.1^{c} (5)	4.1 ± 0.2^{c} (5)
D (+E −Se)	0.15 ± 0.24^{c} (5)	7.35 ± 0.50^{b} (8)	19.0 ± 0.3^{b} (4)	18.2 ± 0.7^{c} (5)	$9.6 \pm 1.0^{a,b}$ (5)	11.2 ± 0.5^{d} (5)
E (−E −Se)	0.24 ± 0.09^{c} (5)	0.82 ± 0.21^{c} (8)	6.3 ± 0.4^{c} (4)	5.3 ± 0.5^{b} (5)	3.5 ± 0.9^{c} (5)	4.2 ± 0.5^{c} (5)
F (Pair-fed)	10.73 ± 0.75^{b} (5)	4.54 ± 0.33^{a} (5)	12.3 ± 0.5^{d} (4)	12.7 ± 0.8^{a} (5)	$8.4 \pm 0.7^{a,b}$ (5)	7.8 ± 0.4^{e} (5)

Each value represents mean ± SEM. Number of rats tested are in parentheses. Means not having a common letter superscript within a column are significantly different ($p < 0.05$).

among the brain regions of rats from different dietary treatments was brain stem > cerebellum > mid-brain = cerebrum.

Incubation of brain homogenates under air for two hr showed dietary effects as manifested by differential susceptibilities of brain regions to lipid peroxidation (Table 4). Cerebrum and mid-brain from rats fed either of the vitamin E-supplemented diets (+E+Se or +E−Se) had significantly lower capacities to produce TBARS than cerebrum and mid-brain from rats fed either of the vitamin E-deficient diets (−E+Se or −E−Se). The effect of doubly supplemented or deficient diets on TBARS relative to control diet was significant only in cerebrum. Dietary vitamin E supplementation had an inhibitory effect on in vitro lipid peroxidation in cerebellum (group D vs groups C and E). Incubation of homogenates showed brain stem of rats treated with any of the diets (except group C) to produce a higher TBARS level than other regions. Brain stem from double-deficient rats showed the highest in vitro TBARS level compared with other dietary treatments. Se-supplemented or deficient diets had no effect on TBARS production under this in vitro condition.

In vitro addition of 0.25 mM ascorbic acid or 0.1 mM Fe^{2+} to the homogenates increased TBARS production several-fold (Tables 5 and 6). Ascorbic acid-induced lipid peroxidation in cerebellum was higher than in other brain regions; brain stem lipid peroxidation was higher than cerebrum and mid-brain in all dietary groups. An effect of dietary treatment on the potential for lipid peroxidation was manifested in cerebrum in response to ascorbic acid challenge. Ascorbic acid-induced lipid peroxidation in cerebrum of rats fed vitamin E-deficient diets (−E+Se and −E−Se) showed significantly higher TBARS than those fed the other diets. A similar trend was observed in mid-brain and cerebellum of −E+Se and −E−Se fed rats and brain stem of −E−Se fed rats but did not reach statistical significance.

In vitro addition of 0.1 mM Fe^{2+} also induced TBARS production in the brain homogenates. However, Fe^{+2} stimulation was greater than ascorbic acid challenge in cerebrum and mid-brain of all dietary groups and as well as in the cerebellum of group A and brain stem of groups B, D, E and F. Vitamin E-deficient rats, particularly those fed −E−Se diet showed the highest in vitro lipid peroxidation in all four brain regions in response to Fe^{2+} challenge. Among the brain regions, cerebellum and brain stem showed the highest responses.

DISCUSSION

Relative to the other tissues, brain contains a low level of vitamin E- and Se-dependent GSH-Px activity (18,29). Furthermore, brain regions vary in their α-T concentration (14–16) and, as shown here, are affected differentially by dietary vitamin E and Se. These components also have been shown to be altered by other physiological and

TABLE 3

Concentration of TBARS Compounds in Brain Regions

Dietary group	Brain region			
	Cerebrum	Mid-brain	Cerebellum	Brain stem
A (Control)	6.7 ± 0.7* (4)	6.7 ± 0.6* (5)	4.3 ± 0.9† (3)	8.7 ± 0.4* (5)
B (+E +Se)	6.2 ± 0.5*,† (5)	5.3 ± 0.6* (4)	7.3 ± 1.3*,† (5)	9.3 ± 1.2† (3)
C (−E +Se)	5.9 ± 0.3* (5)	6.3 ± 0.5* (5)	7.3 ± 1.4* (5)	8.1 ± 0.5* (5)
D (+E −Se)	6.4 ± 0.2* (4)	6.0 ± 0.6* (5)	4.7 ± 0.8* (5)	6.5 ± 1.1* (4)
E (−E −Se)	6.3 ± 0.5* (4)	6.2 ± 0.5* (5)	6.2 ± 1.9* (5)	12.8 ± 6.4* (5)
F (Pair-fed)	5.1 ± 0.5* (4)	5.8 ± 0.5* (5)	5.3 ± 0.9* (4)	10.2 ± 3.3* (4)

Each value represents mean ± SEM of malondialdehyde (nM/mg protein). Number of rats tested are in parentheses. Means within brain regions are not significantly different. Means not having a common * or † superscript within a dietary group are significantly different ($p < 0.05$).

TABLE 4

Concentration of TBARS Compounds in Brain Regions after Incubation at 37 C for Two Hr

Dietary group	Brain region			
	Cerebrum	Mid-brain	Cerebellum	Brain stem
A (Control)	$17.2 \pm 2.0^{b,*,\dagger}$ (4)	$15.7 \pm 2.7^{a,b,*}$ (4)	$15.1 \pm 1.7^{b,c,*}$ (5)	$23.0 \pm 2.0^{a,\dagger}$ (5)
B (+E +Se)	$12.4 \pm 0.9^{c,d,*}$ (5)	$12.0 \pm 0.4^{b,*}$ (3)	$18.0 \pm 7.4^{a,b,c,*}$ (5)	$25.8 \pm 3.7^{a,\dagger}$ (3)
C (−E +Se)	$25.0 \pm 1.5^{a,*}$ (5)	$22.8 \pm 4.3^{a,*}$ (5)	$31.0 \pm 6.3^{a,*}$ (5)	$22.0 \pm 0.9^{a,*}$ (5)
D (+E −Se)	$10.6 \pm 1.7^{d,*}$ (4)	$13.5 \pm 2.1^{b,*}$ (5)	$10.2 \pm 2.0^{c,*}$ (4)	$14.5 \pm 2.6^{a,*}$ (4)
E (−E −Se)	$23.8 \pm 1.0^{a,*}$ (4)	$19.6 \pm 2.3^{a,b,*}$ (5)	$28.6 \pm 6.2^{a,b,*}$ (5)	$29.8 \pm 13.2^{a,*}$ (5)
F (Pair-fed)	$16.2 \pm 1.0^{b,c,*}$ (4)	$19.6 \pm 2.3^{a,b,*}$ (5)	$17.0 \pm 3.3^{a,b,c,*}$ (5)	$25.2 \pm 9.2^{a,*}$ (4)

Each value represents mean ± SEM of malondialdehyde (nM/mg protein). Number of rats tested are in parentheses. Means not having a common letter superscript within a brain region or a common * or † superscript within a dietary group are significantly different ($p < 0.05$).

TABLE 5

Concentration of TBARS Compounds in Brain Regions after Incubation in 0.25 mM Ascorbic Acid at 37 C for Two Hr

Dietary group	Brain region			
	Cerebrum	Mid-brain	Cerebellum	Brain stem
A (Control)	26.5 ± 1.1[b,c] (3)	26.7 ± 7.5[a,b] (5)	48.0 ± 20.7[a] (4)	52.8 ± 5.5[a] (5)
B (+E +Se)	18.3 ± 1.2[c] (5)	21.7 ± 7.7[b] (4)	82.8 ± 34.8[a] (5)	44.9 ± 12.7[a] (3)
C (−E +Se)	54.3 ± 7.0[a] (5)	50.9 ± 13.1[a] (5)	109.0 ± 31.9[a] (5)	55.3 ± 6.4[a] (4)
D (+E −Se)	20.7 ± 1.5[b,c] (4)	22.0 ± 6.0[b] (5)	75.3 ± 35.4[a] (5)	36.7 ± 6.3[a] (4)
E (−E −Se)	51.8 ± 3.5[a] (4)	45.4 ± 7.4[a,b] (5)	119.3 ± 30.7[a] (5)	88.7 ± 35.9[a] (5)
F (Pair-fed)	32.9 ± 4.5[b] (4)	34.4 ± 7.2[a,b] (5)	81.2 ± 30.6[a] (5)	58.0 ± 22.1[a] (4)

Each value represents mean ± SEM of malondialdehyde (nM/mg protein). Number of rats tested are in parentheses. Means not having a common letter superscript within a brain region are significantly different ($p < 0.05$). Means within dietary groups are not significantly different.

TABLE 6

Concentration of TBARS Compounds in Brain Regions after Incubation in 0.1 mM Fe^{2+} at 37 C for Two Hr

Dietary group	Brain region			
	Cerebrum	Mid-brain	Cerebellum	Brain stem
A (Control)	67.3 ± 7.0[a,b,c,*] (4)	54.2 ± 1.2[b,*] (5)	54.5 ± 14.5[a,*] (4)	48.7 ± 3.1[a,*] (5)
B (+E +Se)	60.3 ± 1.7[c,*] (5)	51.9 ± 1.2[b,a] (4)	58.6 ± 14.4[a,*] (5)	45.6 ± 1.1[a,*] (3)
C (−E +Se)	75.0 ± 3.1[a,*] (5)	65.3 ± 3.4[a,*] (4)	87.4 ± 30.0[a,*] (5)	53.6 ± 3.0[a,*] (5)
D (+E −Se)	65.3 ± 0.7[b,c,*] (4)	54.9 ± 2.0[b,*,†] (5)	45.3 ± 6.9[a,†] (5)	45.4 ± 1.7[a,†] (4)
E (−E −Se)	74.4 ± 2.1[a,b,*] (4)	61.2 ± 0.9[a,*] (5)	94.7 ± 33.9[a,*] (5)	101.3 ± 48.6[a,*] (5)
F (Pair-fed)	69.5 ± 0.7[a,b,c,*] (4)	55.3 ± 1.8[b,*] (5)	56.2 ± 12.6[a,*] (5)	63.4 ± 12.7[a,*] (4)

Each value represents Mean ± SEM of malondialdehyde (nM/mg protein). Number of rats tested is expressed in parentheses. Means not having a common letter superscript within a brain region or a common * or † superscript within a dietary group are significantly different ($p < 0.05$).

pathological factors (30,31). The level of α-T in brain regions appears principally affected by dietary vitamin E, an important lipid-soluble antioxidant that influences tissue susceptibility to in vitro and, presumably, in vivo lipid peroxidation.

The changes in α-T concentration induced by dietary treatment vary among the brain regions. For example, in this study, feeding rats with 200 IU of vitamin E for two months increased α-T concentration in cerebellum by 77% (relative to control-fed rats) but only by 36% in cerebrum. Brain tissue has been reported to exhibit a low level of Se-dependent GSH-Px activity relative to other organs (29), and its activity varies among brain regions (17). The biochemical interrelationship between vitamin E and Se is known (32), and their effect on the metabolism of each other and lipid peroxidation has been demonstrated in several tissues (33). However, in this study, supplementation or deficiency of Se did not have any significant effect on α-T level or lipid peroxidation of brain regions during the test period.

It has been reported that susceptibility to lipid peroxidation is markedly influenced by dietary vitamin E and tissue α-T levels (20,32). Brain tissue, due to its high rate of oxygen consumption and high phospholipid content with polyunsaturated fatty acids, is particularly susceptible to peroxidative agents, free radical generating compounds, and lipid peroxidation. However, vitamin E, vitamin C, GSH-Px, superoxide dismutase and other antioxidant defense mechanisms are present in different brain regions with varieties of concentrations and activities (14–17,35–37), working against lipid peroxidation in different brain regions. These cooperative antioxidant defense mechanisms minimize oxidative damage, quench free radical propagation and limit lipofuscin accumulation (6). Although significant differences in α-T concentration were found in the brain regions examined, endogenous levels of lipid peroxidation products, measured as TBARS, did not reflect dietary treatment. Therefore, without in vivo stimulation of lipid peroxidation, measurements of endogenous TBARS of brain regions did not reveal differential effects of dietary vitamin E and Se supplementation or deficiency. However, the presence of relatively low levels of α-T and the detection of relatively higher levels of TBARS in cerebellum and brain stem (Table 2) indicate that these brain regions might be more prone to lipid peroxidation following an in vivo oxidative insult.

Incubation of brain homogenates at 37 C for two hr revealed the influence of dietary treatments on susceptibility to lipid peroxidation. The magnitude of lipid peroxidation was inversely correlated with α-T content. Among the brain regions, brain stem had the highest

capacity for in vitro lipid peroxidation under atmospheric oxygen pressure. Cerebrum and mid-brain regions were least susceptible to lipid peroxidation and possessed the highest concentration of vitamin E.

Addition of ascorbic acid or Fe^{2+} resulted in a several-fold increase of TBARS as reported (38–40). The addition of a low concentration of ascorbic acid reduces oxidized iron and increases lipid peroxidation via initiation of a Fenton-type reaction (41). Differences in the concentration of endogenous iron in brain regions have been partially implicated in the differential susceptibility of brain areas to lipid peroxidation (38). Addition of ascorbic acid to the tissue homogenate upsets the balance between ferric and ferrous iron leading to lipid peroxidation. Our data (Table 5) shows that the cerebellum, relative to other brain regions, had a higher response to the in vitro ascorbic acid challenge, suggesting lipid peroxidation in cerebellum may be related to the high iron content of this brain region. Our data also indicate the cerebellum is more susceptible to ascorbic acid-induced lipid peroxidation, relative to cerebrum and mid-brain even in a state of vitamin E and Se sufficiency. This is in contrast to Noda et al. (39), who found the greatest increase of lipid peroxidation in cerebral cortex, corpus callosum and hippocampus in response to hyperbaric oxygen. They postulated that iron-rich areas in brain generally are vulnerable to iron-catalyzed free radical reactions under hyperbaric oxygen. However, their findings do not closely parallel local brain iron concentrations. The difference between our findings and those of Noda et al. probably is related to the in vivo TBARS metabolism in brain areas after hyperbaric oxygen administration, vs the in vitro ascorbic acid challenge in this study. Thus, the varying iron content of brain tissues may correlate with lipid peroxidation, while the nature of the proxidant challenge may influence the quantitative outcome. It should be noted that ascorbic acid-induced lipid peroxidation still was significantly influenced by dietary intervention in cerebrum and mid-brain where levels of endogenous iron are known to be lower than in cerebellum.

Exogenously added Fe^{2+} further augmented in vitro lipid peroxidation. The addition of Fe^{2+} had a greater stimulatory effect on lipid peroxidation in brain regions with relatively low α-T content and in those regions known to have low dopamine activity. Dopamine has been shown to be effective in ligation of in vitro Fe^{2+} (38). In vitamin E-deficient rats, cerebellum and brain stem, areas with relatively low dopamine activity, showed a slightly higher degree of lipid peroxidation after Fe^{2+} challenge than cerebrum and midbrain.

In this in vitro system, our findings suggest there is a differential susceptibility between brain regions to lipid peroxidation that varies with the peroxidative agent used. In F344 rats, cerebellum and brain stem are more susceptible to lipid peroxidation than other brain regions. This susceptibility is partially affected by the α-T content of these regions, which can be manipulated by dietary vitamin E, but not Se, intervention. Several other endogenous factors, and relative regional distribution of compounds known to be involved in lipid peroxidation processes including total iron, ascorbate, catalase and superoxide dismutase, as well as the composition of fatty acids, phospholipids and neurotransmitters, may contribute to the susceptability of brain regions to lipid peroxidation and the efficacy of dietary vitamin E and Se intervention.

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REFERENCES

1. Nelson, J.S., Fitch, C.D., Fischer, V.W., Brown, G.O. Jr., and Chou, A.C. (1981) *J. Neuropath. Exp. Neurol. 40,* 166–186.
2. Pentshew, A., and Schwarz, K. (1962) *Acta Neuropath. 1,* 313–334.
3. Scott, M.L. (1969) in *Fat Soluble Vitamins* (DeLuca, H.F., and Suttie, G.E., eds.) pp. 335–368, The University of Wisconsin Press, Madison, WI.
4. Towfigi, J. (1981) *Acta Neuropath. 54,* 261–267.
5. Roessman, U., and Frieda, R.L. (1967) *Exp. Neurol. 19,* 508–512.
6. Carpenter, C.S. (1965) *Neurology 15,* 328–332.
7. Tappel, A.L., Fletcher, B., and Deamer, D. (1973) *J. Gerontol. 28,* 415–424.
8. Glees, P., and Masan, M. (1981) in *Age Pigments* (Sohl, R.S., ed.) pp. 303–316, Elsevier, Amsterdam.
9. Muller, D.P.R., Lloyed, J.K., and Wolff, O.M. (1983) *Lancet 1 (8318),* 225–228.
10. Miller, R.G., Davis, C.J.F., Ellingworth, D.R., and Bradley, W. (1980) *Neurology 30,* 1286–1291.
11. Ogunmekan, A.O. (1979) *Am. J. Clin. Nutr. 32,* 2269–2271.
12. Tappel, A.L. (1974) *Am. J. Clin. Nutr. 27,* 960–965.
13. Hoekstra, W.G. (1975) *Fed. Proc. 34,* 2083–2089.
14. Vatassery, G.T., and Younoszai, R. (1978) *Lipids 13,* 828–831.
15. Vatassery, G.T., Angerhofer, C.K., Knox, C.A., and Deshmukh, D.S. (1984) *Biochim. Biophys. Acta 792,* 118–122.
16. Meydani, M., Macauley, J.B., and Blumberg, J.B. (1986) *Lipids 21,* 786–791.
17. Brannan, T.S., Maker, H.S., Weiss, C., and Cohen, G. (1980) *J. Neurochem. 35,* 1013–1014.
18. Kornbrust, D.J., and Mavis, R. (1980) *Lipids 15,* 315–322.
19. Yoshida, S., Busto, R., Watson, B.D., Santiso, M., and Ginsberg, M.D. (1985) *J. Neurochem. 44,* 1593–1601.
20. Bierie, J.G., and Anderson, A.A. (1960) *Arch. Biochem. Biophys. 90,* 105–110.
21. Yasuda, H., Shimado, O., Nakajima, A., and Asano, T. (1981) *J. Neurochem. 37,* 934–938.
22. Meydani, M., Verdon, C.P., and Blumberg, J.B. (1985) *Nutr. Res. 5,* 1227–1236.
23. *Nutrient Requirements of Laboratory Animals* (1978), 3rd edn. (National Research Council, National Academy of Sciences, eds.) pp. 7–37, Washington, DC.
24. Bieri, J.G., Tolliver, T.J., and Catignani, G.L. (1979) *Am. J. Clin. Nutr. 32,* 2143–2149.
25. Paglia, D.E., and Valentine, W.N. (1967) *J. Lab. Clin. Med. 70,* 158–169.
26. Uchiyama, M., and Mihara, M. (1978) *Anal. Biochem. 86,* 271–278.
27. Slater, T.F. (1984) in *Methods in Enzymology* (L. Packer, ed.) Vol. 105, pp. 283–293, Academic Press, Florida.
28. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
29. Lawrence, R.A., and Burk, R.F. (1978) *J. Nutr. 108,* 211–215.
30. Vatessery, G.T., Angerhofer, C.K., and Peterson, F.J. (1984) *J. Neurochem. 42,* 554–558.
31. Vatassery, G.T., Angerhofer, C.K., and Knox, C.A. (1984) *J. Neurochem. 43,* 409–412.
32. Hoekstra, W.G. (1975) *Fed. Proc. 34,* 203–2089.
33. Fischer, W.C., and Whanger, P.D. (1977) *J. Nutr. Sci. Vitaminol. 23,* 273–280.
34. Combs, G.F., Noguchi, T., and Scott, M.L. (1975) *Fed. Proc. 34,* 2090.
35. Seregi, A., Schaefer, A., and Komolos, M. (1978) *Experientia 46,* 1056–1057.
36. Kaplan, E., and Ansari, K. (1984) *Lipids 19,* 784–789.
37. Roy, D., Pahtak, D.N., and Singh, R. (1984) *J. Neurochem. 42,* 628–633.
38. Zaleska, M.M., and Floyd, R.A. (1985) *Neurochem. Res. 10,* 397–410.
39. Noda, Y., McGeer, P.L., and McGeer, E.G. (1982) *Neurobiol. Aging 3,* 173–178.
40. Rehncrona, S., Smith, D.S., Akesson, B., Westerberg, E., and Siesjo, B.K. (1980) *J. Neurochem. 34,* 1630–1638.
41. Walling, C. (1975) *Acc. Chem. Res. 8,* 125–131.

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Isolation and Characterization of Proteolipids from Rat Liver (LSP) and Kidney (KSP)

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In this study, the biochemical characteristics of proteolipids of rat liver and kidney homogenates were compared. In all preparations, the proteo-lipids were eluted at the void volume of the Biogel A-15 m column after gel chromatography, and a molecular weight over 15×10^6 was estimated. Electron microscopy of the concentrated void volume fractions showed globular particles with diameters of 30–100 nm for liver (LSP) and 30–60 nm for kidney (KSP) preparations that might be formed from plasma membrane fragments. After ultracentrifugation in a CsCl gradient, rat LSP and KSP floated in a small density range from 1.16–1.17 g/ml. There was no significant difference between the relative percentages of phospholipids, triglycerides and cholesterol of both proteo-lipids. However, the relative mean amount of total protein in rat KSP (60.4% vs 50.4%) was significantly higher and the content of free fatty acids (4.3% vs 12.6%) significantly lower compared to rat LSP. SDS-PAGE revealed at least 12 protein subunits ranging from 15,000–130,000 in both preparations, but one protein of about M_r 49,000 might represent a liver specific component. The isolated proteo-lipids from rat liver and kidney homogenates showed similar biochemical characteristics as those from human sources, which could explain the known cross-reactivity of antibodies against these preparations.

The putative function of these proteo-lipids is not known, although there is some evidence from human studies that they carry receptor proteins.
Lipids 23, 410–414 (1988).

Cellular and humoral immune reactions against the liver-specific membrane lipoprotein complex (LSP) have been widely reported in patients with liver disease (1,2). LSP is a large, lipid-associated complex containing several antigens, including species-specific and species cross-reactive determinants, of which at least two seem to be liver specific, but their precise identity has not been established (3–5). Immunochemical and electron microscopical evidence suggests that LSP is derived from the liver cell plasma membrane (6–8). An important role of the plasma membrane is to transmit biochemical signals generated by ligand-receptor interaction at the cell surface across the lipid bilayer and into the cell (9). Recently, the hepatic asialo-glycoprotein receptor (hepatic lectin) has been detected as a minor component of liver-specific membrane lipoprotein (10). To our knowledge, this has been the first time that any component of LSP has been positively identified.

Further studies have shown that LSP is a far more complex particle than was first recognized. It has an apparent molecular weight (by gel filtration) of more than 20×10^6, contains phospholipids and triglycerides, and shows multiple protein subunits on SDS-polyacrylamide gel electrophoresis (4). Behrens and Paronetto (11) have demonstrated that kidney extract (KSP) handled in the same manner as liver extract has many similarities to LSP and that antibodies against LSP and KSP give the same immunofluorescence pattern in sections of liver, kidney and stomach. Furthermore, LSP and KSP cross-react in double gel diffusion.

These findings point against a complete organ specificity of LSP in humans in accordance with later studies by Riisom and Diederichsen (12). Hopf et al. tested LSP from different species and showed that rat LSP cross-reacted weakly with human LSP (6). However, Kakumu et al. demonstrated that antiserum to rat LSP, absorbed with normal human plasma, gave a single line on immunodiffusion against purified LSP from rat and human livers. Furthermore, antisera to rat and human LSP reacted almost equally with both rat and human hepatocytes, respectively (1). Whereas biochemical investigations have concentrated on human LSP and more recently KSP, there were to our knowledge no data on the biochemical characterization of these proteo-lipids in rat liver and kidney.

In view of the demonstrated shared antigenic properties, we performed an extensive characterization of these proteo-lipids in rats, which permits a comparative evaluation with the results of the previous studies in humans.

MATERIALS AND METHODS

Preparation of rat liver and kidney membrane lipoproteins. Rat livers and kidneys were obtained from male Sprague-Dawley rats weighing ca. 150 g. The preparation of the membrane lipoproteins was performed at +4 C according to Behrens and Paronetto (11) and McFarlane et al. (4). The liver and kidney tissue was cut into small pieces and washed for five hr in 0.25 M sucrose solution adjusted to pH 8.0 with 0.1 M NaOH, changing the solution every 20 min. For each of the 10 different preparations, 10 rat livers and 20 kidneys were pooled. After crushing the tissue pieces in 0.25 M sucrose solution (wt/vol, 1:1) with an Ultra-Turrax blender, homogenization in a potter homogenizer was performed. The homogenate was ultracentrifugated at 100,000 *g* for one hr using a Beckman 60 Ti-rotor. Two hundred IU/ml penicillin and 10 μg/ml gentamicin were added to the supernatant, and 4 ml were incubated at 20 C with 1.0 μCi of ethanolic [1,2-^{3}H]cholesterol (New England Nuclear, Boston, MA), which is incorporated in the protein lipid complexes and serves as an easily detectable, highly sensitive but not quantitative marker of the lipid moiety. Afterwards, gel chromatography was performed on a 95.0×3.0 cm Biogel A-15 m column with 0.1 M Tris HCl, pH 8.0, containing 0.2 M NaCl and 1 mM Na_2 EDTA as elution buffer.

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Abbreviations: KSP, kidney specific preparations; LSP, liver-specific preparations; PAG, polyacrimide gel; SDS-PAGE, sodium dodecyl sulfate polyacrimide gel electrophoresis.

Usually, 80 to 90 8.4 ml fractions were collected. The absorbance at 280 nm was recorded with a Beckman spectrophotometer. After addition of 5.0 ml Bray-scintillation solution to 0.1 ml aliquots of the fractions, the β-radiation emitted by [1,2-^{3}H]cholesterol was measured in a Betaszint BF 5000 counter. All fractions containing [1,2-^{3}H]cholesterol were pooled and concentrated to about 10% of the original volume using PM 10 membranes (Amicon, Witten, FRG). Further characterization was done by chemical assay, electron microscopy, density gradient ultracentrifugation and SDS-polyacrylamide gel electrophoresis.

Biochemical assays. Protein was measured by the method of Lowry after precipitation of the proteins with trichloracetic acid (13). Total cholesterol and triglyceride determinations were performed enzymatically with commercial test kits (Boehringer Mannheim, FRG) (14,15). The colorimetric NEFA C-test (Wako Chemicals, Osaka, Japan) was used for the quantification of unesterified fatty acids (16). The total phospholipids were determined in chloroform/methanol extracts (3:1, v/v) according to Fiske and Subbarow (17). Further fractionation of the phospholipids was achieved by thin layer chromatography on 20 × 20 cm glass plates coated with silica gel (Merck, Darmstadt, FRG).

Chloroform/methanol/water (65:35:8, v/v/v) was used as solvent system. The separated phospholipids were made visible with iodine vapor and stained individually. Cardiolipin, phosphatidylcholine, sphingomyelin, phosphatidylinositol and ethanolamine glycerophospholipids served as reference substances. Afterwards, a colorimetric phospholipid determination was performed. The accuracy was tested by Precilip E.L. standard sera (Boehringer, Mannheim, FRG).

Density gradient ultracentrifugation. One ml of the sample was added to 3.4 ml of CsCl-solution (density 1.25 g/ml). During 72 hr of ultracentrifugation at 4 C and 50,000 rpm in a Beckman SW-60 rotor, a concave gradient from 1.06 to 1.43 g/ml was established (18). Eleven 0.4 ml fractions were obtained by pipetting from the top. The densities were calculated by weighing 0.2 ml portions. The absorbance at 280 nm and the counts per min were

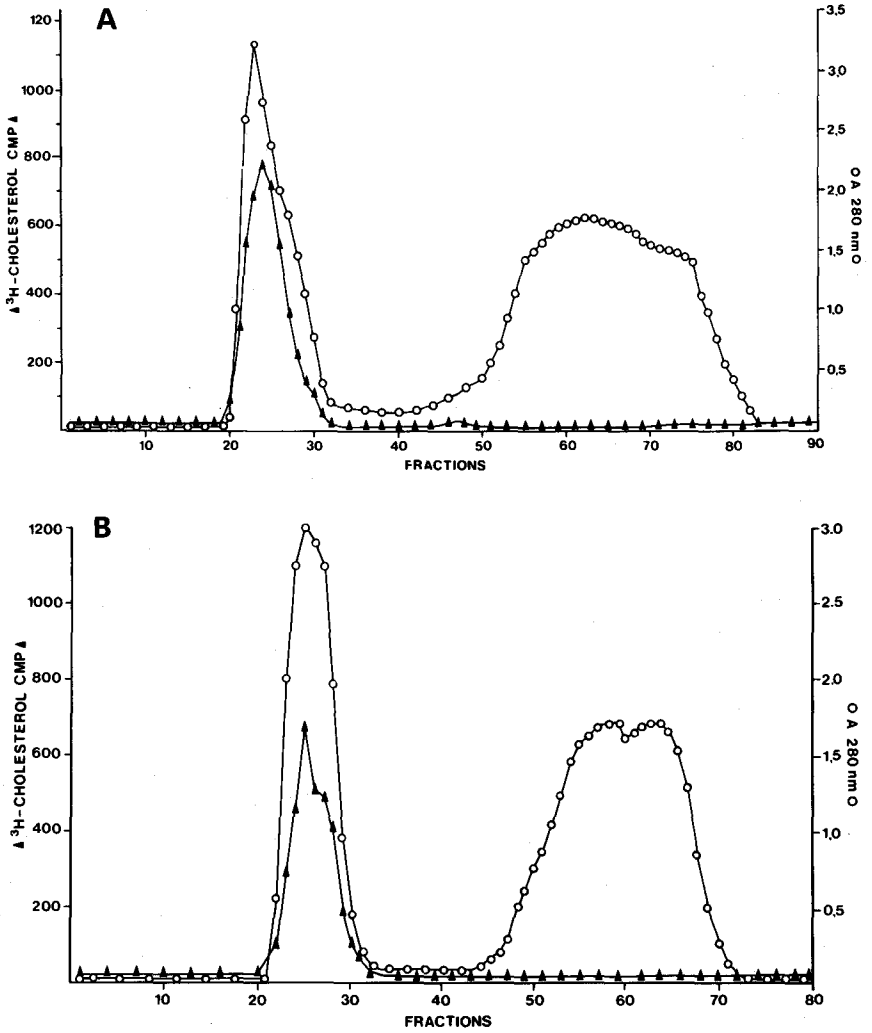

FIG. 1. Typical gel chromatographic elution profile of the concentrated 100,000 g supernatant of liver (A) and kidney homogenates (B) on Biogel A-15 m. Column size 95 × 3.0 cm. Points represent cpm emitted by [1,2-^{2}H]cholesterol (▲) and the absorbance at 280 nm (O) in each collected fraction. V_o, void volume.

measured in each fraction and compared with the position of the macroscopically visible turbid disk of ca. 1-mm height.

Electron microscopy. Electron micrographs were taken with a Siemens 101 electron microscope at 80 kV. The membrane lipoprotein preparations were negatively stained with 2% potassium phosphotungstate, pH 7.0 (19). A small drop of the suspension was placed on a 200 or 300 Formavar carbon-coated grid and examined after drying at room temperature.

SDS-Polyacrylamide gel electrophoresis. For SDS-PAGE, aliquots of the samples were diluted (1:4, v/v) with 37.5 mM Tris HCl buffer (pH 8.8) containing 1 g/dl SDS and 4% 2-mercaptoethanol. After incubation for three min at 95 C, the electrophoretic protein separation was performed in ultrathin (0.36 mm) SDS-polyacrylamide pore gradient gels (T = 4.0–22.5%) on PAG films. The protein subunits were stained with silver nitrate (20). The molecular weights were estimated by comparison with calibration standards (Pharmacia, Freiburg, FRG) according to the method of Weber and Osborne (21).

Statistical analysis. Using mean and standard deviation of the mean for further statistical analysis of our data, Student's t-test was applied.

RESULTS

The elution profile of the 100,000 g liver and kidney supernatants on a Biogel A-15 m column showed rising absorbance at 280 nm, coinciding with elevated counts/min in the first peak as well as high protein absorbance without evidence of radioactivity in the second peak (Fig. 1).

Liver (Fig. 1A, first peak) and kidney (Fig. 1B, first peak) proteo-lipids showed similar elution patterns. Both were eluted in the void volume of the Biogel A-15 m column, indicating a molecular weight of more than 15×10^6.

The determination of the void volume (v_0) had been performed with 10% Dextran 5000 (Serva, Heidelberg, FRG) composing macromolecules of up to 40×10^6.

The chemical analysis of the pooled and concentrated fractions revealed 50.4% protein, 23.2% phospholipids, 12.6% free fatty acids, 11.2% triglycerides and 5.6% total cholesterol (mean values) for liver and 60.4% protein, 20.5% phospholipids, 11.1% triglycerides, 4.7% total cholesterol and 4.3% free fatty acids (mean values) for kidney lipoproteins (Table 1).

The percentage of total protein in rat KSP was significantly ($p < 0.02$) higher and the percentage of free fatty acids significantly ($p < 0.001$) lower compared with the relative amounts in rat LSP. The composition of the phospholipid moiety fractionated by thin layer chromatography is illustrated in Table 2. There were no significant differences for cardiolipin, ethanolamine glycerophospolipids, phosphatidylcholine, phosphatidylinositol and sphingomyelin in both preparations.

An average density of 1.16 g/ml was measured for rat LSP and of 1.17 g/ml for rat KSP by CsCl-gradient ultracentrifugation (Fig. 2A and B). After negative staining, electron microscopy showed globular macromolecules with diameters of 30–100 nm for liver and of 30–60 nm for kidney preparations (Fig. 3A and B). Assuming that one spheric particle on the photograph corresponded to a single molecule, an average molecular weight of 100×10^6 and 50×10^6 was calculated for liver and kidney proteo-lipid complexes, respectively. The particles consisted of at least 12 protein subunits with a molecular weight ranging from 15,000 to 130,000, separated by SDS-PAGE (Fig. 4A and B).

The protein profiles on the gels following SDS-PAGE of rat LSP and rat KSP were similar but show one interesting difference. The protein at about M_r 49,000 appears to be more heavily stained on the liver sample compared with the kidney sample and also may be slightly smaller. The other differences in the electrophoretic profiles of both samples seemed to be caused by varying concentrations of corresponding subunits macroscopically visible by the slightly different intensity of the silver nitrate staining. These visual impressions have been confirmed by a quantitative densitometric scan of these gels.

DISCUSSION

The findings reported here indicate that LSP and KSP could be prepared by standard methods from rat liver and kidney. There were some variations in terms of lipid/protein ratio and the relative amount of the major lipids (phospholipids, free fatty acids, triglycerides, cholesterol) between the 10 different preparations of LSP and KSP. This might be caused by the isolation procedure that composed multiple steps as crushing of the tissue, homogenization, ultracentrifugation, gel chromatography, ultrafiltration and thin layer chromatography. In all preparations, rat LSP and rat KSP were eluted in a single peak at the void volume of the column after gel chromatography. From these results, a molecular weight of rat LSP and rat KSP over 15×10^6 is estimated. However, because of the hydrophobic nature, proteo-lipids may behave atypical on gel filtration, and aggregation of

TABLE 1

Relative Composition of Lipids and Protein in the Rat Liver and Kidney Membrane Lipoproteins of 10 Preparations (Mean ± SEM)

	Liver (%)	Kidney (%)	p-Value
Protein	50.4 ± 2.2	60.4 ± 3.2	<0.02
Phospholipids	23.2 ± 3.9	20.5 ± 2.6	n.s.
Free fatty acids	12.6 ± 1.1	4.3 ± 0.5	<0.001
Triglycerides	11.2 ± 1.8	11.1 ± 2.4	n.s.
Cholesterol	5.6 ± 0.5	4.7 ± 0.7	n.s.

TABLE 2

Relative Composition of Phospholipids in the Rat Liver and Kidney Membrane Lipoproteins of 10 Preparations (Mean ± SEM)

	Liver (%)	Kidney (%)	
Cardiolipin	5.4 ± 1.7	9.3 ± 3.0	n.s.
Ethanolamine glycerophospholipids	23.0 ± 7.6	24.7 ± 1.4	n.s.
Phosphatidylcholine	18.9 ± 7.4	30.8 ± 4.8	n.s.
Phosphatidylinositol	17.2 ± 8.3	19.1 ± 4.2	n.s.
Sphingomyelin	35.5 ± 12.1	15.6 ± 2.2	n.s.

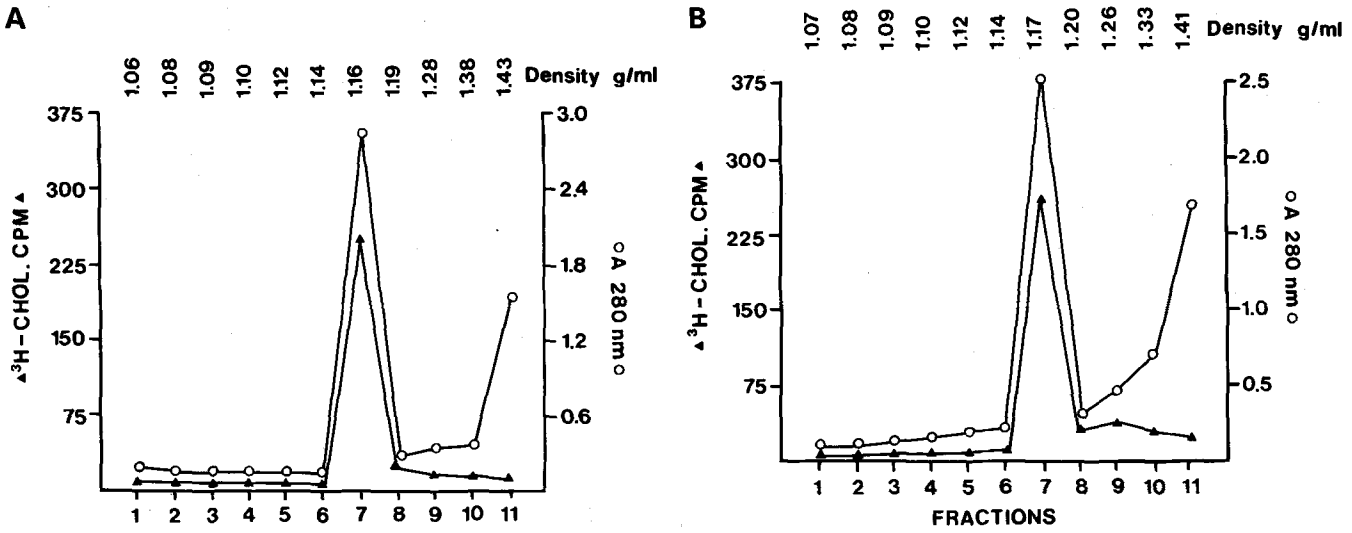

FIG. 2. Density determination of the isolated rat liver (A) and kidney membrane lipoproteins (B) by isopycnic ultracentrifugation in a CsCl gradient (50,000 rpm for 72 hr, 4 C). Points represent cpm emitted by [1,2-^{3}H]cholesterol (▲) and the absorbance at 280 nm (O) recorded in each fraction pipetted from the top.

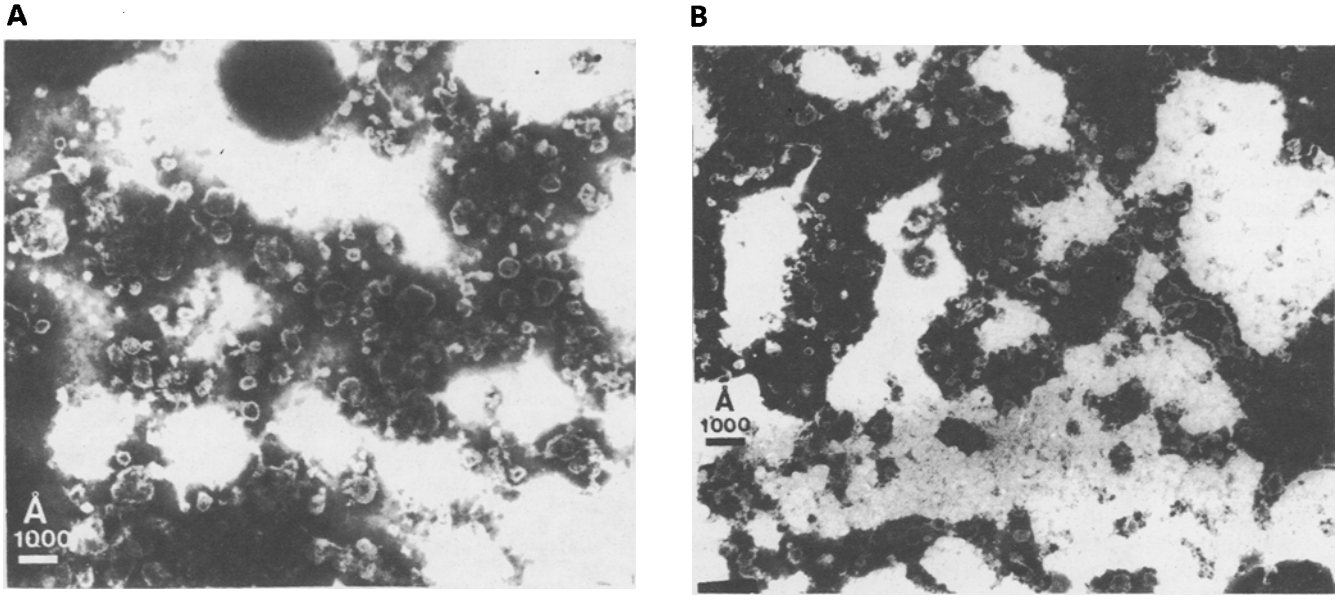

FIG. 3. Electron microscopy of the liver (A) and kidney (B) membrane lipoprotein preparation using a Siemens 101 electron microscope and a negative staining technique with 2% potassium phosphotungstate, pH 7.0 (×60,000).

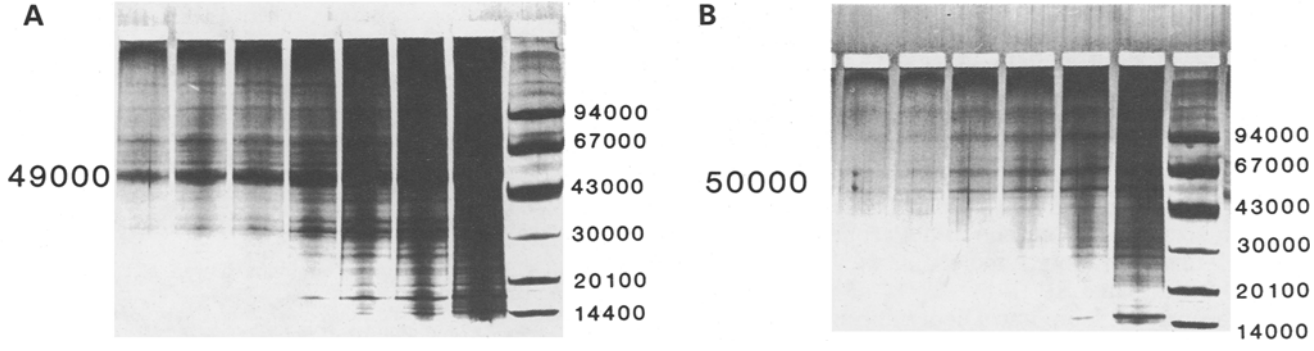

FIG. 4. SDS-PAGE of the liver (A) and kidney (B) membrane lipoprotein preparation on ultrathin (0.36 mm) pore gradient gels (T, 4.0–22.5%) in increasing concentrations. The standard calibration kit is shown in the right lane.

molecules to higher molecular weight complexes is likely. The electron microscopy of the concentrated void volume fractions showed globular particles with diameters of 30–100 nm for liver and 30–60 nm for kidney preparations. These particles could represent vesicles that might have been formed spontaneously from isolated plasma membrane fragments as has been suggested by others from electron microscopical studies on human LSP and KSP (22).

The density gradient ultracentrifugation revealed in all preparations a highly reproducible macroscopically visible turbid disk of ca. 1 mm height with a density between 1.16 g/ml and 1.17 g/ml. This reflects the rather constant protein/lipid ratio of all preparations.

The results of gel chromatography, electron microscopy and density gradient ultracentrifugation showed similar properties of rat LSP and rat KSP. However, further biochemical investigation demonstrated some significant differences between both preparations. The relative mean percentage of total protein in rat KSP (60.4 vs 50.4) was significantly higher and the percentage of free fatty acids (4.3 vs 12.6) significantly lower compared with the relative amounts in rat LSP. However, the free fatty acids might be formed as an artifact during the isolation procedure, and the difference between liver and kidney preparations could be due to different lipolytic activity in these samples. The SDS-PAGE revealed at least 12 protein subunits ranging from 15,000–130,000 in both preparations. One protein at about M_r 49,000 appeared to be more heavily stained on the liver sample compared with the kidney sample and also might be slightly smaller (49,000 vs 50,000). This protein could represent an organ-specific component.

Our results of the quantitative protein-lipid determination in rats were in accordance to the data in humans published by other authors. The average lipid protein ratio of 0.98 in the liver preparations fits in the range of 0.24–1.09 described by Lebwohl and Gerber (8). The mean protein content of 50.4% and the phospholipid moiety of 23.2% showed a similar ratio as reported by McFarlane et al. (4). The phospholipid distribution pattern on cardiolipin, ethanolamine glycerophospholipids, phosphatidylcholine, phosphatidylinositol, sphingomyelin as well as the mean relative amount of triglycerides (11.2%) corresponded to the results of human LSP preparations by Hütteroth and Meyer zum Büschenfelde (5).

Human KSP preparations showed similar gel chromatographic elution patterns and comparable densities compared with our data obtained in rat KSP (11). Furthermore, the lipid protein ratio and the lipid composition resembled those of membrane lipoproteins isolated from human urinary fluid (23). In view of the known cross-reactivity of anti-sera against human LSP and rat LSP, a comparison of the protein profiles of these preparations was of special interest. Studies from McFarlane et al. and Lebwohl and Gerber have indicated that human LSP may be comprised of up to 13 protein subunits of different molecular sizes (4,8). Behrens and Paronetto observed eight protein subunits with molecular weights of 40,000 to 96,000 in human LSP (11).

More recently, Riisom and Diederichsen separated in human LSP and KSP samples four major bands corresponding to molecular weights of 66,000, 60,000, 55,000 and 50,000, and a number of minor protein subunits with molecular weights between 350,000 and 29,000 (12). Our electrophoretic profiles of rat LSP and KSP resembled those of Riisom and Diederichsen very closely, and there was no evidence for a species-specific subunit. These strong similarities could explain the shared antigenic properties of human and rat LSP.

We conclude from our data that large molecular proteo-lipids can be highly reproducibly isolated from rat liver and rat kidney homogenates using standard procedures. Obviously, these proteo-lipids are easily soluble in the cytosol, but immunochemical and electromicroscopical studies of these proteo-lipids from other sources suggest that they constitute fragments of the plasma membrane. There were some differences between rat liver and rat kidney preparations in regard to the relative amounts of protein, free fatty acids and the electrophoretic profile. A 49,000 protein subunit in rat LSP might represent an organ-specific antigen. According to the similar biochemical properties, a close relationship between rat and human LSP and KSP must be assumed. The putative function of these proteo-lipids is not known, although there is some evidence from human studies that they carry receptor proteins for asialo-glycoproteins.

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REFERENCES

1. Kakumu, S., Arakawa, Y., Goji, H., Kashio, T., and Yata, K. (1979) *Gastroenterology 76,* 665–672.
2. Meliconi, R., Perperas, A., Jensen, D., Alberti, A., McFarlane, I.G., Eddlestone, A.L.W.F., and Williams, R. (1982) *Gut 23,* 603–607.
3. Meyer zum Büschenfelde, K.H., and Miescher, P.A. (1972) *Clin. Exp. Immunol. 10,* 89–102.
4. McFarlane, I.G., Wojcicka, B.M., Zucker, G.M., Eddlestone, A.L.W.F., and Williams, R. (1977) *Clin. Exp. Immunol. 27,* 381–390.
5. Hütteroth, T.H., and Meyer zum Büschenfelde, K.H. (1978) *Acta Hepato-Gastroenterol. 25,* 243–253.
6. Hopf, U., Meyer zum Büschenfelde, K.H., and Freudenberg, J. (1974) *Clin. Exp. Immunol. 16,* 117–124.
7. De Kretser, T.A., McFarlane, I.G., Eddlestone, A.L.W.F., and Williams, R. (1980) *Biochem. J. 186,* 679–685.
8. Lebwohl, N., and Gerber, M.A. (1981) *Clin. Exp. Immunol. 46,* 435–442.
9. Walsh, F.S., and Crumpton, M.J. (1977) *Nature 269,* 307–311.
10. McFarlane, I.G., McFarlane, B.M., Major, G.N., Tolley, P., and Williams, R. (1984) *Clin. Exp. Immunol. 55,* 347–354.
11. Behrens, U.J., and Paronetto, F. (1979) *Gastroenterology 77,* 1045–1052.
12. Riisom, K., and Diederichsen, H. (1983) *Gastroenterology 85,* 1271–1276.
13. Lowry, O., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
14. Röschlau, P., Bernt, E., and Gruber, W. (1974) *Z. Klin. Chem. Klin. Biochem. 12,* 403–407.
15. Eggstein, M. (1966) *Klin. Wschr. 44,* 267–273.
16. Okabe, M., Nagashima, K., and Noma, A. (1980) *Clin. Chem. 26,* 1540–1543.
17. Fiske, C.H., and Subbarow, Y. (1925) *J. Biol. Chem. 64,* 375–400.
18. Griffith, O.M. (1976) *Techniques of Preparative Zonal and Continuous Flow Ultracentrifugation,* Application Research Department, Spinco Division, Beckman Instruments Inc., Palo Alto, CA, 4–6.
19. Jones, O.C., and Price, J.M. (1968) *J. Histochem. Cytochem. 16,* 366–370.
20. Merill, C.R., Goldmann, D., and van Keuren, M.L. (1982) *Electrophoresis 3,* 17–23.
21. Weber, K., and Osborn, M. (1969) *J. Biol. Chem. 244,* 4406–4412.
22. McFarlane, I.G. (1984) *Clin. Sci. 67,* 569–578.
23. Jüngst, D., Weiser, H., Siess, E., and Karl, H.J. (1984) *J. Lipid Res. 25,* 655–664.

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Hydroxyacid Derivatives in Human Epidermis

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The principal objective of this investigation was to examine the ω-hydroxyacid derivatives in the cornified layer of human skin. Toward this end, sunburn peelings were collected, and the lipids were extracted with chloroform/methanol. Comparison with authentic standards by thin layer chromatography (TLC) indicated the presence of both N-(ω-acyloxy)acylsphingosine (acylceramide) and ω-acyloxy fatty acid (acyl acid), which accounted for 1.7% and 2.0% of the total lipid, respectively, as estimated by photodensitometry of the charred chromatograms. Each of these lipids was isolated by preparative TLC, hydrolyzed, and the resulting fragments were further analyzed by TLC and by gas liquid chromatography (GLC) of appropriate derivatives. In both of these lipids, the predominant ω-hydroxyacid proved to be the 30-carbon saturated species (56–59% of the total), while linoleate was the major ester-linked fatty acid. Linoleate was more abundant in the acyl acid (38.0%) than in acylceramide (21.4%). These findings represent the first demonstration of acyl acid in human skin and support the proposition that acyl acid, like the other linoleate-rich ω-hydroxyacid derivatives, functions as a molecular rivet in maintaining epidermal structure and function.
Lipids 23, 415–418 (1988).

It has long been recognized that proper differentiation and function of the epidermis is dependent upon an adequate supply of linoleic acid in the diet (1). In the absence of this essential fatty acid, the skin becomes scaly and much more permeable to water. The skin scaliness associated with this dietary deficiency appears to be the result of reduced production of prostaglandins (2); however, the barrier function depends more directly on the availability of linoleate or linoleate-containing molecules in the epidermis (2–4). Several linoleate-containing molecules have been identified recently, and these now are thought to underlie epidermal barrier function (4–9).

In 1978, Gray et al. discovered in pig and human epidermis an unusual glucosylceramide that contains ester-linked linoleic acid (5). Subsequently, our laboratory has shown that this lipid contains very long ω-hydroxyacids amide-linked to sphingosine (6), and the ester-linked linoleate was shown to be attached to the ω-hydroxyl group (7). A structurally related linoleate-rich acylceramide also has been identified in pig and human epidermis (8,10,11). Recently, an analog called acyl acid, in which the sphingosine moiety has been removed, was reported to occur in pig and rat epidermis (9,12). In each of these linoleate-rich epidermal lipids, linoleate is ester-linked to the ω-hydroxyl group of 30- and 32-carbon ω-hydroxyacids.

In this study, we have examined human sunburn peelings, which consist mainly of stratum corneum, to determine whether acyl acid also is present in human skin. Our results demonstrate that acyl acid represents 2.0% of the total lipid extracted from this source. Linoleate composes 38% of the ester-linked fatty acids in this lipid fraction, and the predominant ω-hydroxyacid is the 30-carbon saturated species. The acylceramide isolated from the same material contained a lower proportion of linoleate (21.4%) but had a nearly identical ω-hydroxyacid composition.

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Abbreviations: GLC, gas liquid chromatography; TLC, thin layer chromatography.

MATERIALS AND METHODS

Reference materials. Acylceramide was prepared from pig epidermis as described (8). Pig epidermal acylceramides (8) and giant-ring lactones from donkey sebum (13) served as sources of standard ω-hydroxyacids.

Acyl acid was synthesized by treating 5 mg ω-hydroxyacids prepared from donkey sebum with a 10% molar excess of linoleoyl chloride in 0.25 ml pyridine. After two hr at room temperature, the reaction mixture was chilled in a beaker of crushed ice, and 3 ml chloroform was added. The chloroform solution then was washed three times with 3 ml portions of cold 0.2 N aqueous HCl. The separated chloroform solution was taken to dryness, and acyl acid (1 mg recovered) was purified by preparative thin layer chromatography (TLC) on Silica Gel H using a mobile phase of chloroform/methanol/acetic acid (190:9:1, v/v/v).

Lipid extraction. Four samples of sunburn peelings were dried en vacuo, weighed and extracted for two hr at room temperature in each of three chloroform/methanol mixtures (2:1, 1:1 and 1:2, v/v, v/v and v/v). The combined extracts from each sample were dried under nitrogen and redissolved in a small volume of chloroform/methanol (2:1, v/v). These solutions were filtered through organic-solvent-resistant millipore filters to remove fine particulate material. The solutions then were taken to dryness in tared tubes, and lipid weights were determined. After examination by analytical TLC, the lipid extracts were pooled.

Chemical procedures. Isolated acyl acid and acylceramide were saponified by a slight modification of the procedure described by Gray et al. (5). Basically, the lipid was treated for one hr at 50 C with methanol/chloroform/10 M aqueous NaOH (7:2:1, v/v/v). The reaction mixture was cooled, the pH was adjusted to ca. 4 by addition of aqueous 2 N HCl, and the products were extracted into chloroform.

Amide linkages were hydrolyzed by treatment with 1 M HCl in methanol containing 20 M water at 70 C for 18 hr (14). One volume of water and two volumes of chloroform then were added to the reaction mixture, and after shaking, the chloroform phase was separated. The water/methanol layer then was made alkaline and reextracted with chloroform. The combined chloroform extracts were taken to dryness under a gentle stream of nitrogen.

Isolated long-chain bases were identified as sphingosines by comparison on TLC with an authentic standard (Sigma Chemical Co., St. Louis, MO). This identification was confirmed by gas liquid chromatography (GLC) of

the aldehydes produced by oxidation with sodium periodate as described by Sweeley and Moscatelli (15).

Fatty acids were converted to methyl esters by treatment with 10% BCl_3 in methanol. The methyl esters were extracted into hexane and taken to dryness by evaporation under nitrogen. Acetylation of ω-hydroxy fatty acid methyl esters was achieved by treatment with acetic anhydride/pyridine (1:1, v/v) at room temperature for two hr. Excess reagents were removed by evaporation under nitrogen.

Thin layer chromatography. All analytical TLC employed 0.25 mm-thick layers of Silica Gel G (E.M. Reagents, Darmstadt, West Germany) scribed into 6-mm-wide lanes. Samples were spotted 2 cm from the bottom of the plate. Development with chloroform/methanol/acetic acid (190:9:1, v/v/v) separated the acylceramide and acyl acid from other components. After development, chromatograms were air-dried, sprayed with 50% sulfuric acid and charred at 220 C (16). The charred chromatograms were quantitated by photodensitometry using a Shimadzu model CS-930 photodensitometer.

For preparative TLC, 0.5 mm-thick Silica Gel H (E.M. Reagents, Darmstadt, West Germany) was used. The sample of sunburn lipid was applied as a thin streak 2 cm from the bottom of the plate, which then was developed with chloroform/methanol/acetic acid (190:9:1, v/v/v). After development, plates were sprayed with an ethanolic solution of 2′,7′-dichlorofluorescein. The regions of silica containing the lipids of interest were located under ultraviolet light, scraped from the plate and placed in short, cotton-plugged glass columns. The lipids were then eluted with chloroform/methanol/water (50:50:1, v/v/v).

The products released by saponification of acyl acid and acylceramide were separated by preparative TLC using the same methods described above.

Before analysis by GLC, fatty acid methyl esters were purified by preparative TLC with toluene as the mobile phase. ω-*O*-Acetyl fatty acid methyl esters were purified using a solvent system consisting of hexane/ethyl ether/acetic acid (70:30:1, v/v/v). They then were fractionated according to degree of unsaturation using this same mobile phase and plates containing Silica Gel H impregnated with 10% $AgNO_3$.

Gas liquid chromatography. Fatty acid methyl esters were analyzed using a Varian 3740 gas chromatograph equipped with a 50-meter CP SIL 88 quartz capillary column (Chrompak, Inc., Bridgewater, NJ) and an electronic integrator. The initial column temperature of 160 C was maintained for five min, after which the oven temperature was increased at a rate of 5 C/min until 220 C was reached. Standards included methyl esters of saturated fatty acids ranging from 14 through 24 carbons (kit KF, Applied Science, State College, PA) and a mixture including methyl palmitoleate, methyl oleate and methyl linoleate (kit CE1-62, NuChek-Prep, Elysian, MN).

A 25-meter BP1 quartz capillary column (Scientific Glass Engineering, Inc., Austin, TX) was used at 300 C for analysis of ω-*O*-acetyl fatty acid methyl esters. Previously characterized ω-*O*-acetyl fatty acids prepared (6,8) from pig epidermal lipids and from donkey giant-ring lactones (13) were used as reference materials in these analyses.

RESULTS

Figure 1 shows the separation of acylceramide and acyl acid from other epidermal lipids. Densitometry of similar

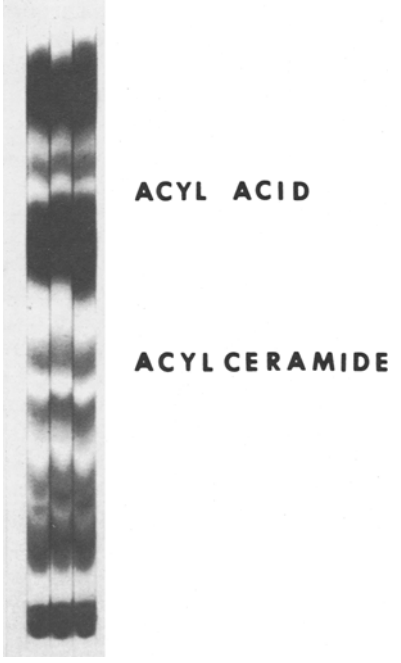

FIG. 1. TLC of the lipids from human sunburn peeling. The spots corresponding to acyl acid and acylceramide are indicated.

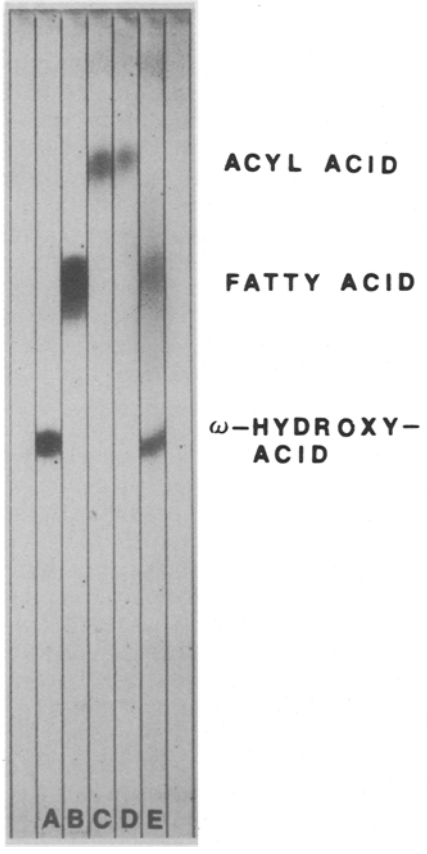

FIG. 2. Characterization of isolated acyl acid. (A) ω-Hydroxyacid prepared from the giant-ring lactones of donkey skin (13). (B) Palmitic acid. (C) Synthetic acyl acid. (D) Acyl acid isolated from sunburn peelings. (E) Products obtained by saponification of human acyl acid.

chromatograms indicated that the acylceramide and acyl acid represent 1.7% and 2.0% of the total lipid, respectively. These values are similar to estimates of acyl acid (9) and acylceramide (8,9) reported for pig stratum corneum.

In Figure 2, acyl acid isolated from the sunburn lipid is compared with synthetic acyl acid and other standards. Also shown, in lane E, are the free fatty acid and ω-hydroxyacid products obtained by saponification of the sunburn acyl acid. The ratio of ω-hydroxyacid to fatty acid in the saponification mixture was estimated by photodensitometry to be 1.6. This observation is in accord with the proposed structure because a charrable carbon ratio of 1.7 would be predicted for an acyl acid in which linoleate is esterified to a 30-carbon ω-hydroxyacid.

The compositions of the fatty acids and ω-hydroxyacids from acylceramide and acyl acid are given in Tables 1 and 2. The major ester-linked fatty acid in each of these lipids was linoleic acid, as shown in Table 1. The predominant ω-hydroxyacid in each lipid is the 30-carbon saturated species. These observations are in good agreement with published findings concerning human acylceramide (10,11). Acyl acid has not been reported to occur in human epidermis.

TABLE 1

Composition of Ester-linked Fatty Acids from Human Acyl Acid and Acylceramide

Fatty acid	Acyl acid (wt %)	Acylceramide (wt %)
14:0	3.8	5.0
14:1	2.3	3.3
15:0	0.7	1.1
16:0	15.4	19.0
16:1	6.3	6.6
17:0	0.7	1.5
18:0	4.8	7.3
18:1	5.7	6.8
18:2	38.0	21.4
20:0	1.7	1.9
22:0	1.9	2.1
24:0	3.8	4.6
Others	14.9	19.4

TABLE 2

Composition of the ω-Hydroxyacids from Acyl Acid and Acylceramide

Hydroxyacid	Acyl acid (wt %)	Acylceramide (wt %)
26:0	3.1	0.8
26:1	0.5	0.2
27:0	0.8	0.7
27:1	0.5	0.2
28:0	3.3	1.6
28:1	0.5	0.2
29:0	2.4	2.6
29:1	0.5	0.1
30:0	58.9	56.5
30:1	1.0	0.6
31:0	5.7	9.1
31:1	0.2	0.2
32:0	10.0	14.7
32:1	10.8	7.9
33:1	0.7	0.4
34:1	4.1	2.8

DISCUSSION

The demonstration that acyl acid is present in human sunburn peelings, although not surprising, is significant in that this is the first report of this unusual lipid in human tissue. Furthermore, in the earlier identification of acyl acid from pig epidermis (9), the ω-hydroxyacid components were examined by TLC only. Indirect evidence was presented indicating that the ω-hydroxyacids in acyl acid are mainly monounsaturated (9). In this work, the ω-hydroxyacids derived from human acyl acid have been examined by TLC on silicic acid, by argentation TLC and by GLC. The predominant ω-hydroxyacid so identified is the 30-carbon saturated species that accounts for 58.9% of the total.

Related to the acyl acid and the acylceramide is a structurally analogous acylglucosylceramide (4–7,9,12), which is present only in the living portion of the epidermis, where it is associated with the epidermal lamellar granules (17). In the later stages of epidermal differentiation, the lamellar granule contents are extruded into the extracellular space, where they fuse in an edge-to-edge manner to produce the multilamellar lipid sheets that fill the intercellular spaces of the keratinized portion of the epidermis (18,19). These extracellular lipid sheets represent the barrier to water loss through the skin (20).

It has been suggested that the acylglucosylceramide and acylceramide act as molecular rivets to hold lipid lamellae in close apposition within the lamellar granules and in the intercellular spaces of the stratum corneum, respectively (4,8). Within this context, it is thought that the ω-hydroxyacyl chain in either of these sphingolipids could span one lipid bilayer, while the ester-linked fatty acid is inserted into an adjacent bilayer, thus riveting the two together. This sort of interaction could promote the flattening and stacking of lipid vesicles to produce the lamellae observed in lamellar granules, and could stabilize the intercellular lamellae within the stratum corneum. Acyl acid also would appear to be geometrically suitable to serve as a molecular rivet in the stratum corneum.

Recently, an alternative hypothesis has appeared in which it is proposed that the linoleate chains of acylceramide and acyl acid are acted on by a lipoxygenase to produce oxy or polyoxy derivatives that modulate epidermal differentiation (12). In this regard, the difference in linoleate content between acylceramide and acyl acid found in this work may reflect differential utilization of these two substrates by this lipoxygenase. Additional work is necessary to determine the relative merits of these two hypotheses and to further document the metabolism of the acylsphingolipids and acyl acid. It should be noted that these two hypotheses are not mutually exclusive. It is possible that acylglucosylceramide, acylceramide and acyl acid serve as molecular rivets, determine physical properties of the epidermal membranes of which they are significant constituents, and are partially converted via lipoxygenase action into regulators of the keratinization process.

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REFERENCES

1. Burr, G.O., and Burr, M.M. (1930) *J. Biol. Chem. 86,* 587-621.
2. Prottey, C. (1977) *Br. J. Dermatol. 97,* 29-38.
3. Elias, P.M., Brown, B.E., and Ziboh, V.A. (1980) *J. Invest. Dermatol. 74,* 230-233.
4. Wertz, P.W., and Downing, D.T. (1982) *Science 217,* 1261-1262.
5. Gray, G.M., White, R.J., and Majer, J.R. (1978) *Biochim. Biophys. Acta 528,* 127-137.
6. Wertz, P.W., and Downing, D.T. (1983) *J. Lipid Res. 24,* 753-758.
7. Abraham, W., Wertz, P.W., and Downing, D.T. (1983) *J. Lipid Res. 26,* 761-766.
8. Wertz, P.W., and Downing, D.T. (1983) *J. Lipid Res. 24,* 759-767.
9. Bowser, P.A., Nugteren, D.H., White, R.J., Houtsmuller, U.M.T., and Prottey, C. (1985) *Biochim. Biophys. Acta 834,* 419-428.
10. Long, S.A., Wertz, P.W., Strauss, J.S., and Downing, D.T. (1985) *Arch. Dermatol. Res. 277,* 284-287.
11. Wertz, P.W., Swartzendruber, D.C., Madison, K.C., and Downing, D.T. (1987) *J. Invest. Dermatol. 89,* 419-425.
12. Nugteren, D.H., Christ-Hazelhof, E., van der Beek, A., and Houtsmuller, U.M.T. (1985) *Biochim. Biophys. Acta 834,* 429-436.
13. Colton, S.W. VI, and Downing, D.T. (1983) *Comp. Biochem. Physiol. 75B,* 429-433.
14. Gaver, R.C., and Sweeley, C.C. (1965) *J. Am. Oil Chem. Soc. 42,* 294-298.
15. Sweeley, C.C., and Moscatelli, E.A. (1959) *J. Lipid Res. 1,* 40-47.
16. Downing, D.T. (1968) *J. Chromatogr. 38,* 91-99.
17. Wertz, P.W., Downing, D.T., Freinkel, R.K., and Traczyk, T.N. (1984) *J. Invest. Dermatol. 83,* 193-195.
18. Matoltsy, A.G. (1976) *J. Invest. Dermatol. 67,* 20-25.
19. Landmann, L. (1986) *J. Invest. Dermatol. 87,* 202-209.
20. Squier, C.A. (1973) *J. Ultrastruct. Res. 43,* 160-177.

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Trifluoperazine Increases Fatty Acid Turnover in Phospholipids in Cultured Human Fibroblasts

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A 24-hr pretreatment of cultured human fibroblasts with trifluoperazine induced a marked increase in incorporation of saturated (stearic, palmitic) and unsaturated (oleic, arachidonic) fatty acids into phospholipids (1.5- to 2-fold for 5.10^{-5} M trifluoperazine). Concomitantly, incorporation into cholesteryl esters was strongly inhibited (20% of control for 5.10^{-5} M trifluoperazine). The drug did not change the phospholipid composition of treated cells. The effect of trifluoperazine on oleic acid incorporation into phospholipids was time-dependent and reached a maximum after a six-hr preincubation with the drug. Trifluoperazine also induced an increase in the rate of chase of oleic acid from the different phospholipid classes. In vitro preincubation of cell-free extracts with trifluoperazine resulted in activation of phospholipid acyltransferases, whereas cholesterol acyltransferase activity was decreased. The rapid effect of trifluoperazine together with its effect on a cell-free system suggests a direct action of this amphiphilic drug on the acyltransferase activities, probably by modification of the structural organization of cellular membranes.
Lipids 23, 419-423 (1988).

Trifluoperazine is an amphiphilic cationic drug of the series of the antipsychotic agents phenothiazines. Levin and Weiss (1,2) first demonstrated that in the presence of calcium, trifluoperazine binds to calmodulin and inhibits its effects. However, it appears that trifluoperazine might exert its action independently of calmodulin. The amphiphilic property of the drug could explain the fact that it partitions readily into cellular membranes (3), thus affecting the activity of membrane-bound enzymes such as calmodulin-independent ATPases (4) or calcium pumps (5).

Phospholipids are important components of cellular membranes, and the composition and turnover of these compounds might be implicated in the regulation of membrane-bound enzymes. It has been shown that trifluoperazine and chlorpromazine inhibit phosphatidylcholine synthesis by incorporation of radioactive choline (6) in HeLa cells. Furthermore, some enzymes of phospholipid metabolism have been shown to be modulated by chlorpromazine, another amphiphilic drug of the phenothiazine series. This drug can activate phosphatidate cytidyltransferase (7) and CDP diacylglycerol inositol phosphatidyltransferase (8), whereas the enzyme phosphatidate phosphohydrolase is inhibited (9,10).

In this work, it is demonstrated that trifluoperazine induced an increase in saturated and unsaturated fatty acids into phospholipids. Concomitantly, incorporation of these fatty acids into cholesteryl esters, taken as internal standard, appeared to be inhibited. In vitro measurement of the acyltransferase activities demonstrated that the drug has a direct effect on the enzyme activities.

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MATERIALS AND METHODS

Materials. Trifluoperazine was from Sigma Chemical Co. (St. Louis, MO); [1-^{14}C]stearic acid (51 mCi/mmol), [1-^{14}C]-palmitic acid (53 mCi/mmol), [1-^{14}C]oleic acid (53 mCi/mmol) and [^{32}P]sodium orthophosphate (20 mCi/mg) were from CEA (Saclay, France); [1-^{14}C]arachidonic acid (58 mCi/mmol), [1-^{14}C]oleoyl-coenzyme A (50 mCi/mmol) and [1-^{14}C]palmitoyl-coenzyme A (58 mCi/mmol) were from Amersham (Buckinghamshire, U.K.); Dulbecco's modified medium with Earle's salts and fetal calf serum were from Gibco (Grand Island, NY). MRC5 human fetal lung fibroblasts were purchased from BioMérieux, Paris (France). Silica Gel F 1500 plates were from Schleicher and Schuell (Dassel, West Germany).

Cell culture. MRC5 fibroblasts were cultured in 60 mm Nunc Petri dishes containing 2 ml Dulbecco MEM medium supplemented with 20 mM Hepes buffer (pH 7.4), 100 units/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) fetal calf serum at 37 C in a humidified atmosphere of 5% CO_2. Experiments were performed on confluent cells seven days after seeding.

Incorporation of fatty acids into phospholipids and cholesteryl esters. After evaporation to dryness under nitrogen, fatty acids were resuspended in a fatty acid-free human serum albumin solution (0.2 g/l). Cells were incubated for one hr (pulse) in culture medium containing radiolabeled fatty acids: stearic acid 39 μM (2 μCi/ml), palmitic acid 38 μM (2 μCi/ml), oleic acid 19 μM (1 μCi/ml) and arachidonic acid 3.4 μM (0.2 μCi/ml). After incorporation, cells were washed four times with a phosphate-buffered solution, pH 7.4, harvested with a rubber policeman and centrifuged. The pellet was resuspended in 9 g/l NaCl. Protein determination was performed on aliquots of the cell suspension by the method of Lowry et al. (11). Phospholipid and neutral lipid separations were performed by thin layer chromatography on silica gel plates in chloroform/methanol/acetic acid/water (50:30:8:4, v/v/v/v) for phospholipids and hexane/diethyl ether/acetic acid (70:30:2, v/v/v) for neutral lipids. The main radiolabeled phospholipids were phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. Aliquots of the cell suspension were applied directly to the plates before chromatography as described (12). After autoradiography, phospholipid and cholesteryl ester spots were cut out and counted by liquid scintillation with an Intertechnique instrument. Results are expressed in pmol of precursor incorporated/mg protein and calculated as percentages of control.

Phospholipid composition. Cells were grown during 48 hr in the presence of 50 μCi/ml [^{32}P]sodium orthophosphate in the presence or absence of the drug. After harvesting, an aliquot of the cell suspension was applied on a silica gel plate and analyzed by bidimensional chromatography: first migration, chloroform/methanol/H_2O (65:25:4, v/v/v); second migration, tetrahydrofuran/methanol/methylal/NH_4OH 2 N (40:20:20:4, v/v/v/v). After autoradiography, the phospholipid spots were cut out and counted in an Intertechnique instrument.

Acyltransferases assay. Acyltransferase activities were measured on sonicated homogenates with a method previously described (13). Each assay, containing 150 μg of protein, phosphate buffer 10^{-1} M (pH 7.4), $MgCl_2$ (5×10^{-3} M), bovine serum-albumin (0.2 mg/ml), was preincubated 15 min at 37 C under gentle shaking in the presence or in the absence of the drug. Final concentrations of trifluoperazine was 10^{-5} to 10^{-3} M, to achieve the same ratio of the absolute number of drug molecules/cell proteins, which was used for in situ experiments (e.g. for in situ incorporation of ^{14}C-oleic acid, 10^{-4} M in 1 ml of medium = 10^{-7} mol of drug/150 μg of cell protein; for in vitro ACAT measurement, 10^{-3} M in 100 μl incubation mixture = 10^{-7} mol of drug/150 μg of cell proteins). After preincubation with the drug, the reaction was initiated by addition of [1-^{14}C]oleoyl coenzyme A or [1-^{14}C]palmitoyl coenzyme A (0.1 μCi) diluted with nonlabeled substrate (final concentration 10^{-5} M). The final incubation volume was 100 μl. The reaction was carried out for five min at 37 C. An aliquot of the incubation mixture then was applied to a silica gel plate, and separation of phospholipids and neutral lipids was achieved by thin layer chromatography. Results are expressed in pmol oleic acid incorporated/five min/mg protein.

RESULTS

The effect of trifluoperazine on saturated and unsaturated fatty acid incorporation into phospholipids and cholesteryl esters is presented in Figure 1. It can be observed that the drug enhanced saturated and unsaturated fatty acid incorporation into phospholipids, whereas the incorporation into cholesteryl esters is inhibited. Furthermore, the two phenomena appeared dose-dependent. After treatment with 5.10^{-5} M of the drug, incorporation into phospholipids reached 150%–220% of control, depending on the fatty acid, and incorporation into cholesteryl esters decreased to about 20% of control. It is of note that the distribution of radioactivity among the different labeled phospholipid classes (phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol) is not affected by the presence of trifluoperazine (Table 1). Thus, it might be concluded that the drug equally stimulated fatty acid incorporation into the different phospholipid classes, independently of the polar head group moiety. It must be noted that one-hr incorporation represented a pulse period for fibroblasts taken in the described conditions. The consumption of the radiolabeled fatty acids during this period accounted for approximately 1% of total for stearic and palmitic acids, 2% for oleic acid and 9% for arachidonic acid.

One of the questions raised is whether the composition of phospholipids is modified in treated cells. By isotopic equilibrium technique utilizing [^{32}P]orthophosphate incorporation into the polar head groups of phospholipids, it was found that the composition in percentages of phospholipids is not affected (Table 2), nor is the phospholipid/protein ratio (results not shown). So, if the phospholipid composition is unchanged and if incorporation of fatty acids is stimulated, then it means that the turnover of fatty acids is accelerated in phospholipids. Pulse-chase experiments then were performed, and it was found that in our system trifluoperazine increases the rate of chase of oleic acid from labeled phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Table 3).

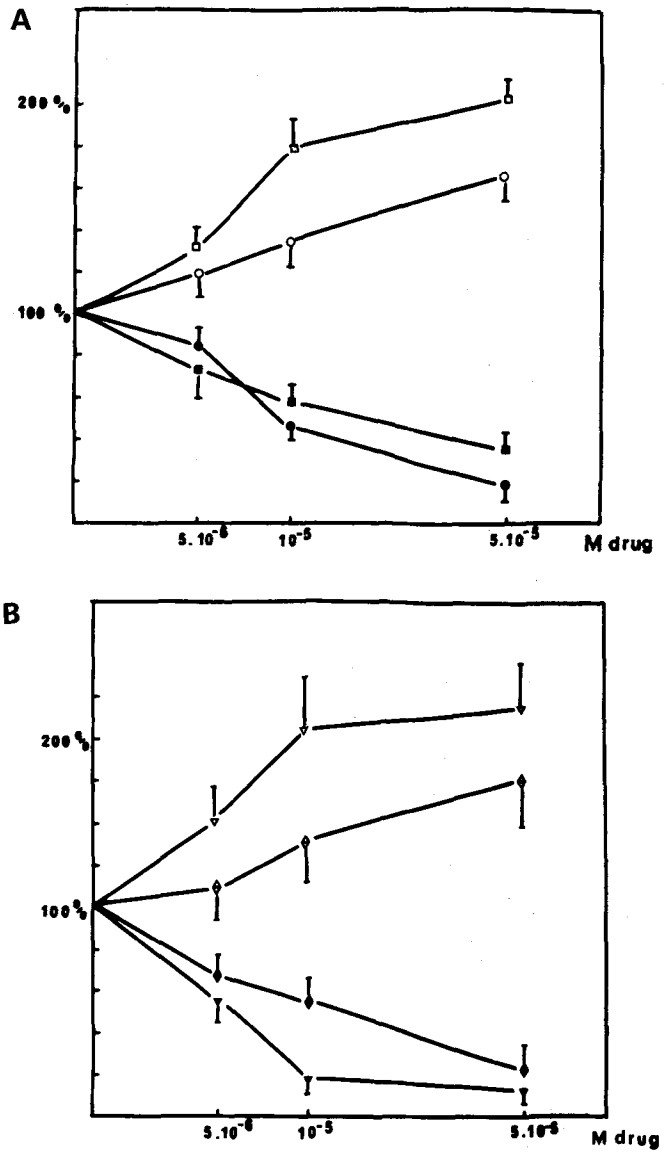

FIG. 1. Effect of trifluoperazine on incorporation of saturated (A) and unsaturated (B) fatty acids into phospholipids and cholesteryl esters. Incorporation was performed during one hr after a 24-hr pretreatment with the drug. Results are expressed as percentages of control (means of 3 experiments ± SD). The absolute values are in pmol of the fatty acid incorporated/mg proteins, stearic acid: (■), cholesteryl esters 110, (□), phospholipids 3857; palmitic acid: (●), cholesteryl esters 48, (○), phospholipids 2718; oleic acid: (▼), cholesteryl esters 143, (▽), phospholipids 4623; arachidonic acid: (◆), cholesteryl esters 46, (◇), phospholipids 2854.

We further investigated the time-dependency of oleic acid incorporation after pretreatment with trifluoperazine, and the results are presented in Table 4. It appeared that the effect of the drug on oleic acid incorporation into phospholipids and cholesteryl esters is rapid and can be observed as soon as two hr after incubation with the drug. The effect increased with preincubation time up to six hr incubation, when incorporation into phospholipids reached 190% of the control value.

TABLE 1

Effects of Trifluoperazine (TFP) on Incorporation of Fatty Acids into Different Lipid Classes

Precursor	Addition	Cholesteryl esters	Triacyl-glycerols	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-inositol
Stearic acid	None	110 ± 6	903 ± 110	2198 ± 181	926 ± 72	733 ± 79
	TFP 10^{-5} M	63 ± 7	560 ± 52	4286 ± 318	1713 ± 154	1393 ± 126
Palmitic acid	None	48 ± 3	737 ± 67	2310 ± 183	163 ± 9	272 ± 26
	TFP 10^{-5} M	22 ± 2	594 ± 48	3150 ± 293	217 ± 15	384 ± 39
Oleic acid	None	143 ± 12	931 ± 82	3144 ± 296	647 ± 58	832 ± 76
	TFP 10^{-5} M	29 ± 3	830 ± 57	6298 ± 520	1242 ± 97	1647 ± 124
Arachidonic acid	None	46 ± 4	58 ± 5	1341 ± 111	913 ± 72	600 ± 46
	TFP 10^{-5} M	25 ± 2	42 ± 3	1609 ± 134	1141 ± 92	752 ± 53

Incorporation was performed one hr after 24-hr pretreatment with trifluoperazine 10^{-5} M. Results are expressed in pmol fatty acid incorporated/mg proteins. Means of three experiments ± SD.

TABLE 2

Phospholipid Composition of Human Cultured Fibroblasts in the Presence or Absence of Trifluoperazine

Addition	Sphingomyelin	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylinositol
None	13.6 ± 1.5	61.1 ± 2.3	17.6 ± 1.9	7.7 ± 0.9
TFP 10^{-5} M	13.2 ± 0.9	59.8 ± 3.5	18.2 ± 1.2	8.8 ± 1.0

Cells were incubated 48 hr with ^{32}P sodium orthophosphate 50 μCi/ml, and phospholipids were analyzed by bi-dimensional chromatography. Results are expressed in percentages of total radioactivity.

TABLE 3

Effect of Trifluoperazine on Pulse Chase Experiments with Oleic Acid

Addition	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylinositol
None	89% (±7%)	87% (±6%)	76% (±7%)
TFP 10^{-5} M	62% (±5%)	65% (±6%)	57% (±5%)

Cells were pretreated 24 hr with the drug before radiolabeled oleic acid addition during one hr (pulse). After washing, cells were further incubated in nonlabeled medium in the presence or absence of the drug for six hr (chase). The values obtained after the pulse period are taken as 100% for each phospholipid. Results are expressed in percentages of radioactivity found after the chase period for each phospholipid. Means of three experiments ± SD.

TABLE 4

Kinetics of the Effect of Trifluoperazine on Oleic Acid Incorporation into Phospholipids and Cholesteryl Esters

Addition	Preincubation time	Oleic acid incorporated into (percentages of control)	
		Cholesteryl esters	Phospholipids
		100%	100%
Trifluoperazine 10^{-5} M	2 hr	68 ± 5	133 ± 9
	4 hr	37 ± 4	171 ± 11
	6 hr	27 ± 3	192 ± 11
	17 hr	24 ± 3	215 ± 18
	24 hr	22 ± 2	220 ± 19

Cells were preincubated for the indicated time with trifluoperazine 10^{-5} M. [1-^{14}C]Oleic acid, 1 μCi/ml, was then added and incroporaton followed during one hr. Results are expressed in percentages of control. Means of three experiments ± SD.

TABLE 5

Effect of Trifluoperazine Preincubation with Cell-free Extracts on Palmitic Acid and Oleic Acid Transfer to Different Lipid Classes

Substrate	Addition	Cholesteryl esters	Phospholipids			
			Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-inositol	Total
Palmitoyl-coenzyme A	None	48 ± 4	897 ± 72	238 ± 14	332 ± 24	1467 (100%)
	TFP 10^{-5} M	37 ± 3	912 ± 81	252 ± 18	376 ± 27	1540 (105%
	TFP 10^{-4} M	18 ± 3	1103 ± 92	283 ± 21	418 ± 28	1804 (123%)
	TFP 10^{-3} M	6 ± 2	1300 ± 112	326 ± 25	560 ± 49	2186 (149%)
Oleoyl-coenzyme A	None	58 ± 5	978 ± 115	172 ± 21	374 ± 42	1524 (100%)
	TFP 10^{-5} M	49 ± 5	1056 ± 95	189 ± 19	408 ± 36	1653 (108%)
	TFP 10^{-4} M	31 ± 4	1223 ± 114	203 ± 23	430 ± 49	1856 (122%)
	TFP 10^{-3} M	9 ± 2	1492 ± 163	272 ± 32	598 ± 54	2362 (155%)

Cell extracts were preincubated 15 min with trifluoperazine at the indicated concentration before measurement of acyltransferase activities with [1-^{14}C]oleoyl-coenzyme A or [1-^{14}C]palmitoyl-coenzyme A as described in the text. Results are expressed in pmol incorporated/five min/mg protein. Means of three experiments ± SD.

The short-term effect of trifluoperazine on oleic acid incorporation into phospholipids suggests a direct effect of the drug on enzyme activity. On the other hand, as it is well-known that phospholipid acyltransferases are specific for saturated and unsaturated fatty acids, the activity of acyltransferases were measured on cell-free extracts after in vitro preincubation with the drug with two different substrates: palmitoyl-coenzyme A and oleoyl-coenzyme A. Results from Table 5 clearly indicate that a 15-min preincubation of cell extract with the drug is sufficient to induce an increase in phospholipid acyltransferase activities and a decrease in cholesterol acyltransferase activity. Again, it can be noted that incorporation into the different phospholipid classes is equally stimulated. Preincubation with 10^{-3} M trifluoperazine resulted in about 1.5-fold increase of phospholipid acyltransferase activities, whereas cholesterol acyltransferase decreased to 15% of control.

DISCUSSION

A 24-hr pretreatment of cultured fibroblasts results in stimulation of fatty acid incorporation into phospholipids, namely phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. It is of note that while the acidic phospholipid phosphatidylinositol derives from cytidine-diphosphodiacylglycerol, the two other phospholipids, phosphatidylcholine and phosphatidylethanolamine, have a common precursor, diacylglycerol. Trifluoperazine stimulating fatty acid incorporation into the three phospholipids suggests that the drug exerts its action through the acyltransferase activity itself rather than through the availability of phospholipid precursors. Another line of evidence arises from the fact that incorporation of fatty acids into triacylglycerols, another neutral lipid derived from diacylglycerol, is either poorly affected or decreased by the drug under the same conditions (Table 1). The described effects of trifluoperazine also are observed in our system with another amphiphilic cationic drug, chlorpromazine (results not shown). Martin et al. (14) recently reported that chlorpromazine inhibited the synthesis of phosphatidylcholine and triacylglycerols in isolated rat hepatocytes by using radiolabeled oleate and glycerol. These authors concluded that chlorpromazine partially acts through the rate of conversion of phosphatidate to the common precursor, diacylglycerol. In our system, however, incorporation of oleate into phospholipids is enhanced by trifluoperazine and chlorpromazine. One reason that might explain the discrepancy of the results is the difference in the cell types utilized by these authors and by ourselves. This hypothesis is further supported by the fact that phosphatidylcholine synthesis from choline is sensitive to phenothiazines in HeLa cells (15) and not in rat hepatocytes (16).

The isotopic equilibrium method demonstrated that the phospholipid composition itself is unchanged in trifluoperazine-treated cells. As within the cells, phospholipids undergo a cycle of deacylation-reacylation reactions, it thus appears that trifluoperazine accelerates the rate of turnover of saturated and unsaturated fatty acids in the phospholipid molecules. This is confirmed by pulse-chase experiments performed with oleic acid (Table 3). In this regard, the question of an eventual modification of the fatty acid composition of the different phospholipid classes in trifluoperazine-treated cells might be raised.

The rapid effect of trifluoperazine suggests a direct activation of the phospholipid acyltransferase enzyme. This is confirmed by in vitro measurement of the acyltransferase activities in cell-free extracts after preincubation with the drug. It was demonstrated that the enzyme acyl coenzyme A:cholesterol-acyltransferase is inhibited, while the phospholipid acyltransferases measured either with oleoyl-coenzyme A or with palmitoyl-coenzyme A are stimulated. The mechanism whereby phenothiazines modulate these enzyme activities has to be elucidated. It is well-known that phenothiazines bind to the hydrophobic domain of calmodulin (17,18) and thus block the action of Ca^{2+}-calmodulin. Thus, the hypothesis of a regulation by Ca^{2+}-calmodulin of the acyltransferase activities must be considered. However, besides this effect on calmodulin, phenothiazines might independently act on cell metabolism, especially by means of membrane-bound enzymes, by modifying the physico-chemical characteristics of membranes such as membrane potential (19)

or by modulating the conformation of membrane structure (20). Another explanation might be that trifluoperazine can regulate the subcellular partition of membrane-bound enzymes. Such a regulation mechanism has been reported for some enzymes of phospholipid synthesis such as CTP:phosphocholine-cytidylyltransferase (21) and phosphatidate phosphohydrolase (22).

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REFERENCES

1. Levin, R.M., and Weiss, B. (1977) *Mol. Pharmacol. 13,* 690–697.
2. Levin, R.M., and Weiss, B. (1979) *J. Pharmacol. Exp. Ther. 208,* 454–459.
3. Landry, Y., Amellal, M., and Ruckstuhl, M. (1981) *Biochem. Pharmacol. 30,* 2031–2032.
4. Luthra, M.G. (1982) *Biochim. Biophys. Acta 692,* 271–277.
5. Hirata, M., Suematsu, E., and Koga, T. (1982) *Biochem. Biophys. Res. Commun. 105,* 1176–1181.
6. Pelech, S.L., and Vance, D.E. (1984) *Biochim. Biophys. Acta 795,* 441–446.
7. Sturton, R.G., and Brindley, D.N. (1977) *Biochem. J. 162,* 25–32.
8. Zborowski, J., and Brindley, D.N. (1983) *Biochim. Biophys. Acta 751,* 81–89.
9. Brindley, D.N., Allan, D., and Michell, R.H. (1975) *J. Pharm. Pharmacol. 27,* 462–464.
10. Bowley, M., Cooling, J., Burditt, S.L., and Brindley, D.N. (1977) *Biochem. J. 165,* 447–454.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
12. Mazière, C., Mazière, J.C., Mora, L., and Polonovski, J. (1987) *J. Biochem. Biophys. Methods 14,* 267–272.
13. Mazière, C., Mazière, J.C., Mora, L., and Polonovski, J. (1982) *Biochim. Biophys. Acta 712,* 712–715.
14. Martin, A., Hopewell, R., Martin-Sanz, P., Morgan, J.E., and Brindley, D.N. (1986) *Biochim. Biophys. Acta 876,* 581–591.
15. Pelech, S.L., Jetha, F., and Vance, D.E. (1983) *FEBS Lett. 158,* 89–92.
16. Pelech, S.L., and Vance, D.E. (1984) *Biochim. Biophys. Acta 795,* 441–446.
17. Rainteau, D., Wolf, C., Béréziat, G., and Polonovski, J. (1984) *Biochem. J. 221,* 659–663.
18. Roufogalis, B.D. (1981) *Biochem. Biophys. Res. Commun. 98,* 607–613.
19. Snelling, R., and Nicholls, D. (1984) *J. Neurochem. 42,* 1552–1557.
20. Verkleij, de Maagd, R., Leunissen-Bijvelt, J., and De Kruijff, B. (1982) *Biochim. Biophys. Acta 684,* 255–262.
21. Pelech, S.L., Cook, H.W., Paddon, H.B., and Vance, D.E. (1984) *Biochim. Biophys. Acta 795,* 433–440.
22. Hopewell, R., Martin-Sang, P., Martin, A., Saxton, J., and Brindley, D. (1985) *Biochem. J. 232,* 485–491.

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Disposition and Metabolism of Pristane in Rat

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The fate of pristane (2,6,10,14-tetramethylpentadecane), a widespread isoprenoid hydrocarbon, has been studied in rats after a single per os administration of ^{3}H-labeled pristane. The balance study showed an extensive fecal excretion (66%) mainly as unchanged hydrocarbon, whereas about 14% of ingested pristane was excreted in urine as pristane metabolites and tritiated water. After one wk, 8.3% of the ingested ^{3}H still was stored in the carcass, and radioactive distribution in tissues and organs showed a preferential incorporation into adipose tissue and liver. Over 75% of the radioactivity stored in the carcass was associated with pristane metabolites and tritiated water. Tissue metabolites were characterized by thin layer chromatography, gas chromatography and mass spectrometric analyses. Four metabolites were identified: pristan-1-ol, pristane-2-ol, pristanic acid and 4,8,12-trimethyltridecanoic acid. These demonstrate that this isoprenoid hydrocarbon undergoes subterminal hydroxylation or terminal oxidation followed by the classical β-oxidation process. Incorporation of metabolites in phospholipids and more particularly in the phosphatidylserine fraction has been observed and is discussed.
***Lipids 23,* 424-429 (1988).**

Much of the information on the fate of saturated hydrocarbons in vertebrates deal with the metabolism and disposition of n-alkanes; less has been reported for branched-alkanes. Among the latter, pristane (2,6,10,14-tetramethylpentadecane) and phytane, both of which may be of either biogenic or petrogenic origin, usually are abundant in living matter. Pristane was found at concentrations of 80-1070 ppm in fish oil (1) and 2-52 ppm in human and mammal tissues (2).

As early as 1967, these residues were considered as resulting from the difference between the rates of input and removal, thus it was suggested that branched-chain hydrocarbons are not inert in the animal tissues but are metabolized rapidly (2). It was reported several years later that rats are able to oxidize phytane to alcohols and acids (3); nevertheless, no information was available regarding biotransformation of pristane in higher animals.

The purpose of this study was to establish the metabolic balance of pristane in rats and to characterize pristane metabolites isolated from the whole body and the liver. Furthermore, because unusual long chain alcohol or branched-chain fatty acids may result from the ω-oxidation of pristane, as described for microorganisms (4,5), incorporation of metabolites into lipid compartments was investigated.

MATERIALS AND METHODS

Chemicals. Pristane was purchased from Aldrich-Chimie (Strasbourg, France). [^{3}H]Pristane (142 MBq/μmol) was prepared by direct contact of pristane with tritium gas (Wilzbach method) at the C.E.A. (Saclay, France), then purified by chromatography on a silica gel column (Kieselgel 60, Merck), using hexane as solvent. The purity, as evaluated by gas chromatography (GC) and reverse phase high pressure liquid chromatography (HPLC), was higher than 96%.

Tetramethylpendadecanoic acid was the gift of J. Flanzy (INRA, CNRZ, Jouy en Josas, France).

Animal experiments. Wistar male rats weighing about 200 g were accustomed for one wk before studies to a semisynthetic diet (casein 18%, wheat starch 39%, sucrose 24%, peanut oil 8%, cellulose 3%, minerals and vitamins 8%). The night before the experimental diet was given, the animals were fed moderately.

(a) Experiment 1: metabolic balance. Three animals were held in individual metabolism cages. At the beginning of the experiment, each animal received a 59.2 MBq [^{3}H]pristane dose previously incorporated in the peanut oil of the diet. After complete consumption of the impregnated feed, free access to the untreated semisynthetic diet again was given to the animals.

Urine and feces were collected separately and daily for seven consecutive days. A few drops of HCl (1 N) were added to urine for storage. Animals were killed on the seventh day after dosing, and various tissues and organs were sampled.

After careful removal of the gastrointestinal tract contents, the remainder of the carcasses were minced using a domestic machine. Feces and aliquots of homogenized carcass were saponified with ethanolic KOH, then the unsaponifiable fraction was extracted with hexane. Hydrocarbons were isolated by column chromatography on activated silica by hexane elution.

Urine analysis was performed by successive extractions with hexane and ethyl acetate.

(b) Experiment 2: metabolites identification and incorporation of ^{3}H into lipids. Administration of labeled pristane (20 MBq) occurred as described in experiment 1, except that before the incorporation of the hydrocarbon in the peanut oil, the specific radioactivity of [^{3}H]pristane was adjusted to 278 KBq/μmol with unlabeled pristane (Aldrich-chimie).

Lipid and metabolite analyses were performed as described in the following paragraphs.

Hepatic lipid analysis. The distribution of radioactivity in hepatic tissues was studied after lipid extraction according to the procedure of Folch (6). Total lipids were separated into particular classes on silica cartridges (Sep-Pak, Waters, Milford, MA) by successive hexane, chloroform and methanol elutions, yielding hydrocarbon, neutral lipid and phospholipid fractions, respectively (7). Phospholipids then were separated into different components by HPLC (8). Purity of fractions was tested by thin layer chromatography (TLC) on Silica Gel G using chloroform/methanol/acetic acid/water (100:55:16:4, v/v/v/v) as solvent system.

Metabolites analysis. (a) Isolation procedures. Livers were homogenized in 10 ml of water with a Polytron homogenizer at room temperature. The same procedure

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Abbreviations: TMS, trimethylsilyl; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high pressure liquid chromatography; TLC, thin layer chromatography.

was used for carcasses, after careful removal of the gastrointestinal contents. Homogenates were saponified with ethanolic KOH; then the unsaponifiable fraction was extracted with hexane. Hydrocarbons and unsaponifiable metabolites were isolated from this fraction by chromatography on a silica gel column (Kieselgel 60, Merck, Darmstad, FRG) by successive hexane and chloroform/methanol (2:1, v/v) elutions. After acidification by HCl, fatty acids were extracted with hexane from the aqueous phase. A flow chart of the different steps is summarized in Figure 1.

(b) Analytical procedures. (1) TLC: Kieselgel 60, 250 μ chromatographic plates (Merck, Darmstadt, Germany) were used with hexane/ethyl ether/acetic acid (80:20:1, v/v/v) as the solvent system. Radioactive areas were located with a radiochromatogram scanner LB2832, Berthold (Wildbad, FRG). (2) GC: A Hewlett Packard 5710A gas chromatograph equipped with a 2m × 1/8" stainless steel column packed with 3% Dexsil 300 on 100/120 Supelcoport (Supelco Inc., Bellefonte, PA) was used, programmed from 120 C to 340 C at 10 C/min. For methyl ester analyses by radio-GC, the gas chromatograph was connected to a reactor (RGC 170 Bodenseewerk Perkin-Elmer Co. Ueberlingen, Germany), in which tritium gas was measured with a proportional counting tube after hydrogenative cracking of the ^{3}H-labeled compounds. (3) GC/MS: A Hewlett Packard model 5992 B gas chromatograph/mass spectrometer equipped with a 15 m × 0.2 mm flexible fused silica column with OV1 coating (Hewlett-Packard) was used, programmed from 150 C to 250 C at 10 C/min and with a He (carrier gas) flow rate of 1 ml/min.

Derivatization. Fatty acid methyl esters were obtained from acids by esterification with methanol/benzene/sulfuric acid (17:2:1, v/v/v) for three hr at 96 C.

Fatty alcohols, having been extracted (fraction II) and purified by TLC, were acetylated with acetic anhydride/pyridine (4:1, v/v) at ambient temperature during three hr.

Trimethylsilyl (TMS) derivatives were prepared by treating the various compounds with BSTFA + 1% TMCS or with BSTFA/TSIM/TMCS (100:50:1, v/v/v) mixtures (Pierce Chemical Co., Rockford, IL) in anhydrous acetonitrile for four hr at 70 C.

Radioactivity measurements. Aliquots of urine samples were counted directly by liquid scintillation counting (Packard Minaxi 4000 apparatus). The total radioactivity of tissues, organs and carcasses was measured after combustion of aliquots (200–500 mg) in an oxidizer (Oxymat, Intertechnique, Kontron, Trappes, France) that ensures trapping of ^3H_2O in a scintillation mixture (Triox, Kontron).

Estimation of tritiated water was obtained by comparing the radioactivity in fresh and lyophilized samples after it has been established that no loss of unchanged hydrocarbon or metabolites occurred during the freeze-drying process.

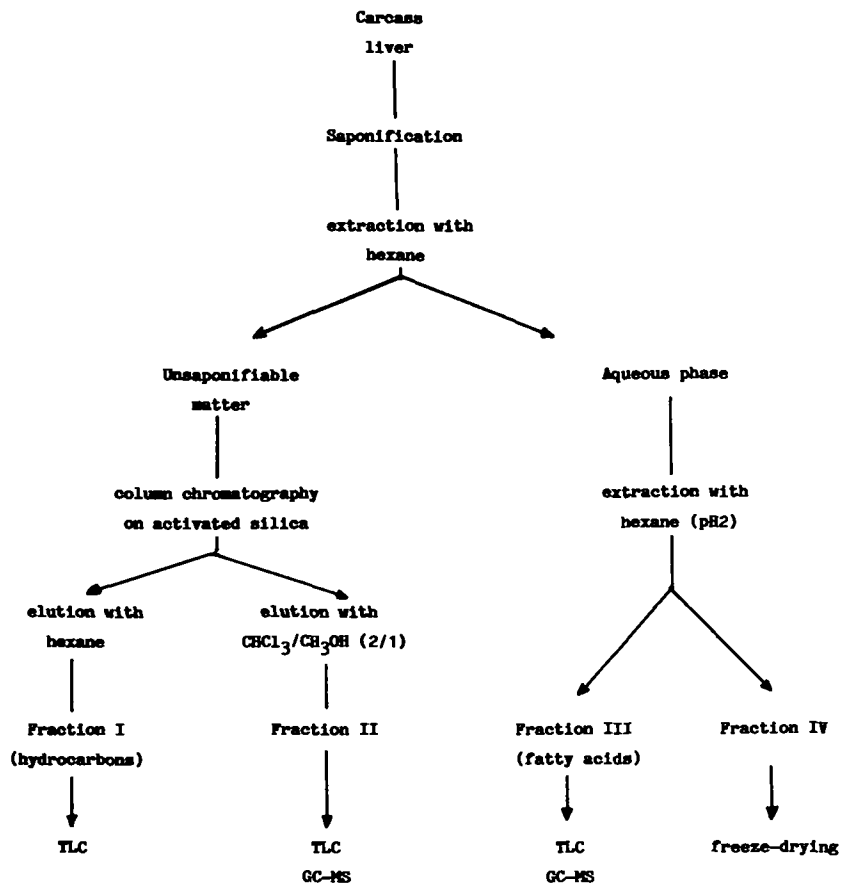

FIG. 1. Schematic diagram for the analysis of tissues from rats dietary exposed to [^{3}H]pristane.

RESULTS

The specific activity of ^{3}H-pristane measured before administration and after the residual hydrocarbon has been isolated from tissues indicates that hydrogen exchange did not occur to any significant extent (Fig. 2).

Balance study. The distribution of ^{3}H in excreta and whole carcass of rat following a single dose of [^{3}H]pristane is reported in Table 1. This table shows that radioactivity was excreted mainly in feces, whereas 8.26% of the ingested ^{3}H was still stored in the carcass after one wk. Only 23.5% of the radioactivity present in the carcass was due to pristane. The occurrence of metabolites (20.5%) and tritiated water (56%) underline the intensity of metabolic transformations of pristane in rat.

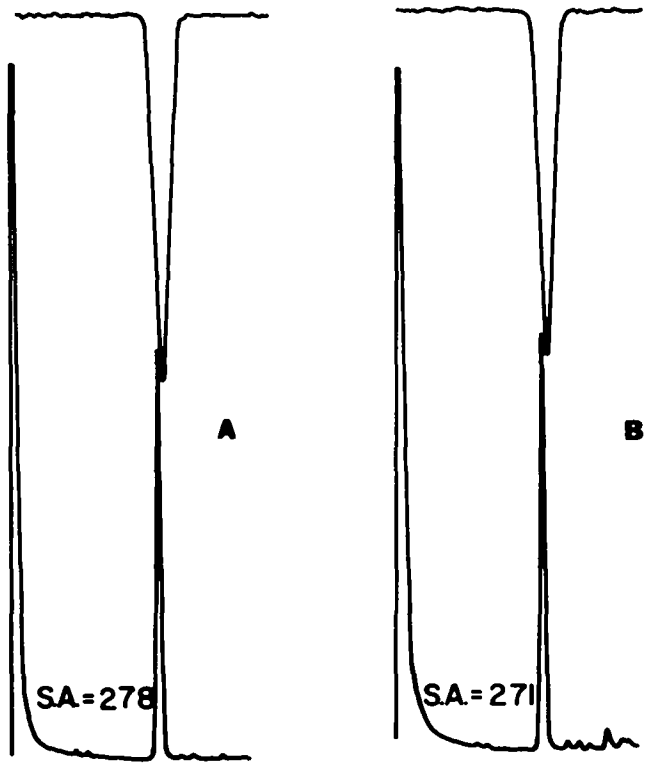

FIG. 2. Radio gas chromatography of hydrocarbon fed (A) and of hydrocarbon in carcass (B): upper trace, radioactivity; lower trace, mass; S.A., specific activity (KBq/μmol).

TABLE 1

Distribution of ^{3}H in Carcass and Excreta Seven Days after Oral Ingestion of [^{3}H]pristane (Expressed as % of Administered Dose)[a]

	Tritiated water	Metabolites	Hydrocarbon	Total radioactivity
Carcass	4.65	1.70	1.91	8.26 ± 0.81
Feces	1.50	10.78	53.94	66.22 ± 1.64
Urine	3.88	10.98	n.d.[b]	14.86 ± 1.25
Total recovery				89.34 ± 2.02

[a][^{3}H]pristane dose was 59.2 MBq. Values are means ± SD from three animals.

[b]n.d., Not detected.

The kinetics of the distribution of the radioactivity in feces indicates that by this route, excretion reached its maximum level between 24 hr and 48 hr (Fig. 3). A more detailed analysis of the kinetics of depletion of fecal radioactivity over the seven-day period shows a rapid elimination of pristane, whereas metabolite excretion occurs more slowly than with hydrocarbons.

The excretion of radioactivity was much lower in urine than in feces (Table 1). The data presented in Figure 4 show a rapid urinary elimination; more than 65% of urinary ^{3}H was excreted during the first 48 hr. During the first few days, radioactivity excretion occurred mainly as pristane metabolites; then, ^{3}H was equally distributed between metabolites and tritiated water. No trace of hydrocarbon was detected in urine.

The disposition of ^{3}H among body tissues and organs, as measured one wk after oral administration of [^{3}H]-pristane, is shown in Table 2: specific activities levels in perigastric adipose tissue and in liver were higher than those in kidney, lung, spleen and heart.

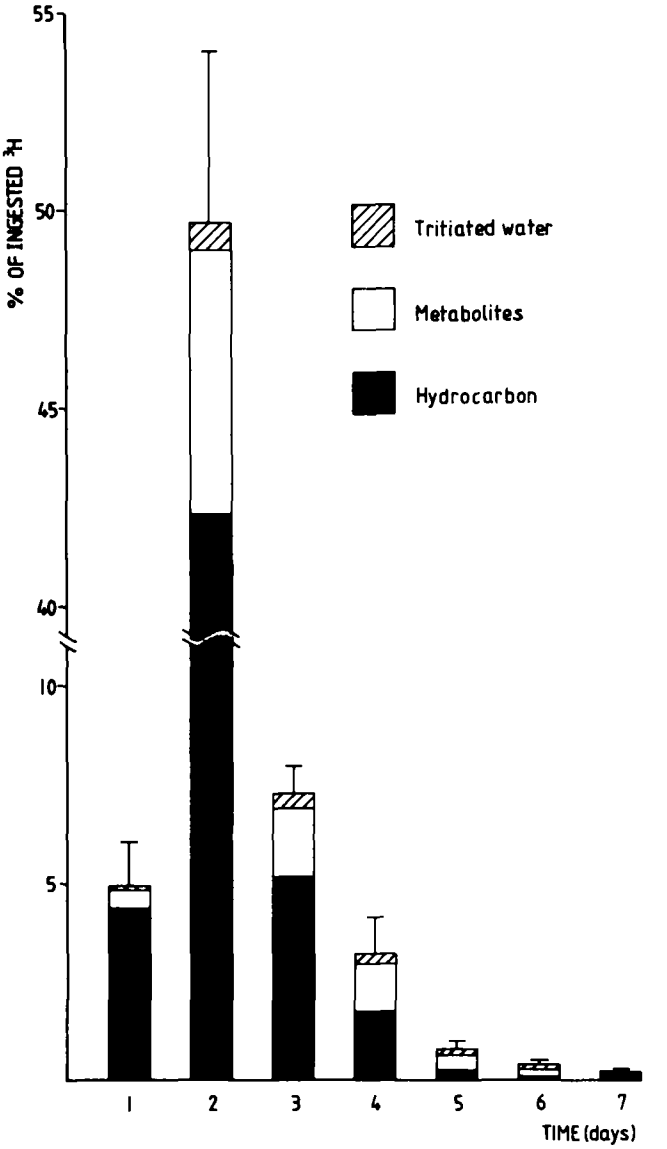

FIG. 3. Evolution and distribution of ^{3}H in feces of rats fed [^{3}H]pristane.

Distribution of ^3H in hepatic lipids. The incorporation of tritium into hepatic lipids was studied six hr, 24 hr and one wk after ingestion of [^{3}H]pristane (Table 3). The radioactivity was associated mainly with the hydrocarbon fraction throughout the experiment. However, labeling of neutral lipids and phospholipids fraction already was significant at six hr; at this time, specific activity of neutral lipids was higher than that of phospholipids.

However, after one wk labeling of neutral lipids and phospholipids was 900 and 1010 Bq/g respectively. The separation of the different classes of phospholipids (Table 4) revealed that most of the radioactivity was bound to phosphatidylcholine. However, when expressed as Bq/g the major incorporation of ^{3}H was detected in phosphatidylserine.

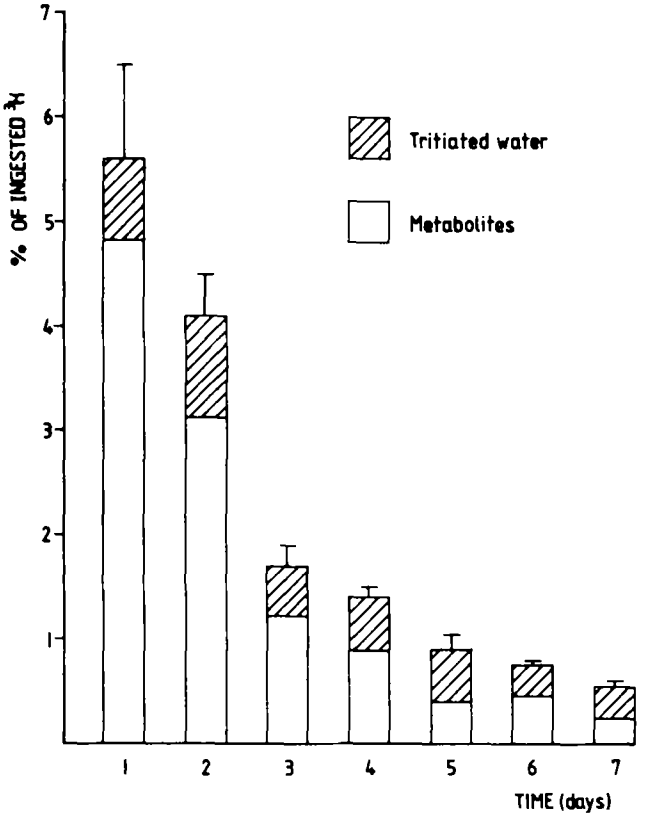

FIG. 4. Evolution and distribution of ^{3}H in urine of rats fed [^{3}H]pristane.

Identification of metabolites. Liver and carcass metabolites were analyzed after saponification. The different fractions (see Fig. 1) were analyzed by TLC followed by radiochromatogram scanning. Confirmation of the identity of metabolites was obtained through GC/MS analysis.

(a) Fraction I. The labeled compound detected in this fraction possessed TLC and GC properties identical with those of pristane. This fraction represented the major part of liver radioactivity eight hr after [^{3}H]pristane administration (Table 5). It accounted for 23.6% of carcass radioactivity after seven days.

(b) Fraction II. ^{3}H detected in this fraction accounted for 10.9% and 13.7% of carcass and liver radioactivity, respectively. TLC analysis of this fraction revealed a radioactive zone at R_f 0.25. This R_f-value was similar to that of a long-chain alcohol. Acetylation of this fraction, in addition to unchanged material, resulted in a less polar product with chromatographic mobility similar to that of a long-chain alcohol acetate. GC analysis of fraction II confirmed it to be a mixture of two metabolites with retention times of 10.23 (A) and 11.61 min (B).

TABLE 2

Distribution of ^{3}H in Tissues Seven Days after Oral Administration of [^{3}H]pristane[a]

	(% of ingested ^{3}H) × 10^3	Kbq/g
Liver	508 ± 57	31.0 ± 5.5
Adipose tissue	—[b]	38.7 ± 9.4
Kidney	56 ± 8	16.9 ± 3.0
Spleen	27 ± 3	21.6 ± 3.6
Lung	53 ± 15	16.2 ± 4.5
Heart	24 ± 5	16.3 ± 4.1
Remaining carcass	7592 ± 735	19.5 ± 2.8
Total	8260 ± 810	

[a][^{3}H]pristane dose was 59.2 MBq. Values are means ± SD from three animals.
[b]Not estimated.

TABLE 3

Distribution of Radioactivity in Liver Lipids after Ingestion of 20 mg [^{3}H]pristane[a]

	Six hr		24 Hr		Seven days	
	S.A.	%	S.A.	%	S.A.	%
Hydrocarbons	1,930	92.5	671	90.4	43	95.8
Neutral lipids	96	4.6	35	4.7	0.9	2
Phospholipids	60	2.9	36	4.9	1	2.2
Total incorporation (KBq)	876		290		23	

[a]Data are expressed as specific activity (KBq/g) and % of total incorporation at each time point. Values are from pooled samples from two animals.

TABLE 4

Distribution of Radioactivity in Liver Phospholipids of Rat, Following a Single Dose of 20 mg of [^{3}H]pristane[a]

	Six hr		24 Hr		Seven days	
Phospholipids	Percent of total ^{3}H	Bq/g	Percent of total ^{3}H	Bq/g	Percent of total ^{3}H	Bq/g
PE	19.7	35,710	26.6	30,850	28.1	2,810
PI	8.2	51,600	7.4	35,300	8.5	2,950
PS	6.3	97,950	4.1	41,760	7.1	6,410
PC	61.4	48,480	59.6	28,900	49	2,000
Sph	4.3	58,580	2.3	22,630	7.1	3,650

[a]Values are from pooled samples from two animals.
PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; Sph, sphingomyelin.

When analyzed by GC-MS, the mass spectra from (A) and (B) showed significant ions at m/e (266(M-18) and 59(C_3H_7O), indicating that these metabolites are both hydroxylated derivatives of pristane.

The TMS derivative of (A) exhibited characteristic fragment ions at m/e 341 (M-15) and 131 ($(CH_3)_2$-CO-TMS), which are characteristic of the TMS ether of a tertiary alcohol. The TMS derivative of (B) presented the same significant ion at m/e 341 but also a characteristic signal at m/e 103, indicating a primary alcohol function. These mass spectral results suggest that metabolite (A) was pristan-2-ol and metabolite (B) pristan-1-ol (Fig. 5). Quantitative analysis by TLC and GC revealed that pristan-2-ol accounted for 88% of the mixture of the two pristanols in carcass.

(c) Fraction III. Radioactivity detected in this fraction was slightly lower than in Fraction II (Table 5). The TLC behavior of both underivatized (R_f 0.4) and methylated (R_f 0.8) Fraction III material suggested the presence of fatty acids.

After successive elimination of unsaturated and linear fatty acids (9), radio-GC analysis of the liver fatty acid methyl esters showed that the radioactivity was bound to an unusual fatty acid (metabolite C). Radio-GC of carcass fatty acid methyl esters resulted in two labeled peaks. The major one exhibited the same retention time as metabolite C (10.49 min). The retention time of the second product (metabolite D) was 8.35 min.

Study of the methyl ester of metabolite (C) by GC-MS exhibited an apparent M^+ at m/e 312 and significant fragments at m/e 88 (rearrangement ion: -$CH(CH_3)$-CO-O-CH_3) and 101 (CH_2-$CH(CH_3)$-CO-O-CH_3). The comparison between this mass spectrum and the fragmentation of 2,6,10,14-tetramethylpentadecanoic acid methyl ester indicated that metabolite C was pristanic acid (Fig. 5). The mass spectrum of the methyl ester of metabolite D showed a molecular ion at m/e 270 and characteristic peaks at 87 (CH_2-CH_2-CO-O-CH_3) and 74 (CH_2-CO-O-CH_3), indicating that (D) corresponded to 4,8,12-trimethyltridecanoic acid (Fig. 5).

(d) Fraction IV. In liver and carcass, it has been shown after lyophilization that ^3H detected in this fraction was associated with tritiated water. This was the principal labeled compound detected in carcass (Table 5).

TABLE 5

Distribution of Radioactivity in the Different Analytical Fractions from Carcass and Liver[a]

Fractions	Carcass[b]	Liver[c]
I (Hydrocarbons)	23.6	62.3
II (Fatty alcohols)	10.9	13.7
III (Fatty acids)	8.5	11.1
IV (Tritiated water)	57.0	12.9

Reported values are expressed as percentage of total radioactivity and are from three pooled samples.

[a]For detail, see Figure 1.

[b]Seven days after [^3H]pristane administration.

[c]Eight hr after [^3H]pristane administration.

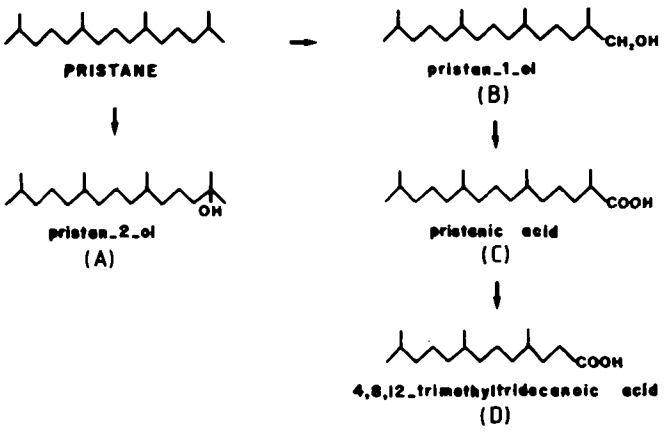

FIG. 5. Proposed metabolic pathways of pristane in the rat.

DISCUSSION

This study was initiated to obtain more detailed knowledge of the fate of alkanes in mammals. Previous studies on alkane metabolism have been conducted using n-heptadecane and dodecylcyclohexane as models of normal and monocyclic alkanes, respectively (10,11). Our results clearly show that absorption, storage and oxidation of pristane occur in the same way as for these two alkanes.

In feces, more than 80% of the radioactivity was attributable to pristane. Several authors have shown that no hydrocarbon oxidation by the microbial flora occurs in rat gut (3,12). Moreover, in a study concerning the biliary excretion of hydrocarbons in rat, we have observed that no elimination of unchanged hydrocarbon occurred via this route (Tulliez and Le Bon, unpublished results). Therefore, we could estimate the digestive absorption of pristane as the dose ingested minus the amount of pristane in feces, i.e. in this study a digestibility level of 46%.

This value is lower than those observed for other alkanes such as n-heptadecane (10) and dodecylcyclohexane (11), but is close to the results previously reported for a 15-mg dose of pristane (13).

An appreciable amount of radioactivity remained in the carcass beyond seven days. As expected, considering the lipophilic nature of hydrocarbons, the highest levels of labeling were found in adipose tissue and liver.

The fact that after one wk, more than 75% of the radioactivity stored in the carcass was associated with pristane metabolites and tritiated water demonstrates that pristane is thoroughly metabolized in rat. Fatty alcohols are the first step of this biotransformation, as confirmed by the studies of PerduDurand and Tulliez (unpublished results) who studied in vitro oxidation of pristane. Detection of two isomers of pristanol shows that pristane hydroxylation may occur in ω or ω-1 position of the alkyl chain. The subterminal hydroxylation (ω-1) leads to pristan-2-ol that could be a metabolic dead end; hydroxylation on a tertiary carbon would prevent any further oxidative step and may explain the substantial amounts of this metabolite in tissues of rat. This must be related to the findings of Albro and Thomas (3) who observed, in studying the metabolism of phytane in rat, the presence of phytan-2-ol in rat liver.

The subsequent oxidation of pristan-1-ol to pristanic acid may explain the low amount of primary pristanol detected in carcass and liver. Pristanic acid undergoes the classical fatty acid β-oxidation pathway, as shown by the presence of 4,8,12-trimethyltridecanoic acid and tritiated water in tissues.

The isolated metabolites demonstrate that methyl substituents in position 2,6,10,14 do not prevent the classical mechanisms of terminal and β-oxidation mentioned by several authors for n-alkanes (10,14). However, it must be noted that the metabolic pathways of pristane in rat are limited in comparison with microorganisms in which oxidation at both ends (15,16) or attack on carbon 3 (17) may occur in addition to the terminal oxidation.

Liver lipid analysis demonstrates the occurrence of pristane metabolites in neutral lipids and phospholipids. As reported in labeled heptadecane and dodecylcyclohexane studies (10,11), the greater deposition of radioactivity in phospholipids occurred in the phosphatidylcholine fraction. However, measurements of specific activities show a preferential incorporation of pristane metabolites in phosphatidylserine. Specific role of this phospholipid in some membrane enzymatic processes has been demonstrated (18). Thus, it will be of interest to investigate the possible interactions of branched-chain fatty acids with the biochemical mechanisms in which the structural lipids are involved. The assessment of toxicological significance of these uncommon fatty acids must be questioned because it has been shown that high levels (>5%) of a branched-chain fatty acids mixture in rat diet resulted in growth inhibition and accumulation of these fatty acids in tissue lipids (19).

Nevertheless, the present work demonstrates that, at low levels in the diet, pristane is rapidly metabolized by terminal oxidation and then, as for dietary fatty acids, by successive β-oxidation reactions. These results complete previous data on alkane metabolism and give a better understanding of their metabolic utilization by mammals.

REFERENCES

1. Ackman, R.G. (1971) *Lipids 6,* 520-522.
2. Avigan, J., Milne, G.W.A., and Highet, R.J. (1967) *Biochim. Biophys. Acta 144,* 127-131.
3. Albro, P.W., and Thomas, R.O. (1974) *Biochim. Biophys. Acta 372,* 1-14.
4. Cox, R.E., Maxwell, J.R., Ackman, R.G., and Hooper, S.N. (1974) *Biochim. Biophys. Acta 360,* 166-173.
5. Singer, H.E., and Finnerty, W.R. (1984) in *Petroleum Microbiology* (Atlas, R.M., ed.), pp. 1-59, MacMillan Publishing Co., New York.
6. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
7. Juaneda, P., and Rocquelin, G. (1985) *Lipids 20,* 40-41.
8. Juaneda, P., and Rocquelin, G. (1986) *Lipids 21,* 239-240.
9. Tulliez, J., and Peleran, J.C. (1977) *FEBS Lett. 75,* 120-122.
10. Tulliez, J.E., and Bories, G.F. (1978) *Lipids 13,* 110-113.
11. Tulliez, J.E., and Bories, G.F. (1979) *Lipids 14,* 292-297.
12. Albro, P.W., and Fishbein, L. (1970) *Biochim. Biophys. Acta 219,* 437-446.
13. Tulliez, J., and Bories, G. (1975) *Ann. Nutr. Alim. 29,* 201-211.
14. McCarthy, R.D. (1964) *Biochim. Biophys. Acta 84,* 74-79.
15. McKenna, E.J., and Kallio, R.E. (1971) *Proc. Natl. Acad. Sci. USA 68,* 1552-1554.
16. Pirnik, M.P., Atlas, R.M., and Bartha, R. (1974) *J. Bacteriol. 119,* 868-878.
17. Rontani, J.F., Bertrand, J.C., Blanc, F., and Giusti, G. (1986) *Mar. Chem. 18,* 9-16.
18. Roelofsen, B., and Van Deenen, L.L.M. (1973) *Eur. J. Biochem. 40,* 245-257.
19. Smith, A., Lough, A.K., and Earl, C.R.A. (1982) *J. Sci. Fd. Agric. 33,* 421-430.

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Oxidation of 1-*O*-(Alk-1-enyl)-2,3-Di-*O*-Acylglycerols: Models for Plasmalogen Oxidation[1]

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Alk-1-enyl diacyl glycerides, model compounds for plasmalogen lipids, were synthesized for use as substrates in oxidation studies. The neutral plasmalogen glycerides prepared included 1-*O*-(hexadec-1-enyl)-2,3-di-*O*-stearoylglycerol (*4a*) and 1-*O*-(hexadec-1-enyl)-2-*O*-linoleoyl-3-*O*-stearoylglycerol (*4b*). Oxidative disappearance of these glycerides was followed directly by high performance liquid chromatography (HPLC). The extent of oxidation of the side chains of these glycerides, i.e. the alkenyl ether and fatty acyl functions, was monitored by conversion of the side chains to dimethylacetal and methyl ester by methanolysis, followed by subsequent gas chromatography (GC) of the methanolysis products. Both analytical approaches show that the alkenyl ether function of neat *4a* oxidizes more slowly than neat ethyl linoleate. However, the rate of alkenyl ether loss from *4a* is accelerated in the presence of added ethyl linoleate. Moreover, when the linoleoyl group is incorporated into the 2-position of the alkenyl glyceride, as in *4b*, the rate of loss of the alkenyl group was shown to be similar to the rate of loss of the linoleoyl group. These results suggest that oxidation of plasmalogen glycerides should not be ignored as a factor that contributes to the oxidative instability of animal tissue.
***Lipids 23,* 430–434 (1988).**

In muscle and other animal tissue, phosphatides are not confined just to the diacyl variety. Substantial amounts of the long-chain groups at the glycerol *sn*-1-position are in the form of alkyl ethers, and as much as a third of the choline glycerophosphatides and two-thirds of the ethanolamine phosphatides are plasmalogens, i.e. *sn*-1-(alk-1-enyl) ethers (1). Studies of lipid oxidation in foods typically are focused on the role of autoxidation of polyunsaturated acyl groups in phosphatides (generally at the *sn*-2 position), especially with regard to the course of such oxidation in the development of rancidity in meat (2–5). On the other hand, only very recently has attention been focused on the autoxidation of the double bond in plasmalogens as a potential contributor to such rancidity (6). In the latter report, it was shown that a model enol ether, ethyl hexadec-1-enyl ether, was prone to in vitro autoxidation, especially in the presence of polyunsaturated esters. Other studies have shown that natural plasmalogens are subject to in vivo peroxidation by enzymatic systems (7–9) and that alk-1-enyl ethers are subject to attack by singlet oxygen (10,11). This study was undertaken to investigate whether the alk-1-enyl functionality of neutral plasmalogen glycerides undergoes autoxidation as do simple vinyl ethers and to assess the effect of polyunsaturated acyl functionality, both inter- and intramolecular, on the course of such autoxidation.

MATERIALS AND METHODS

Hexadecanal was prepared from hexadecanol (Aldrich Chemical Co., Milwaukee, WI) by oxidation using dimethylsulfoxide (DMSO) and dicyclohexylcarbodiimide (Sigma Chemical Co., St. Louis, MO) according to the method of Fenselau and Moffatt (12). Glycerol-1,2-carbonate was prepared by the ester interchange of D,L-1-*O*-benzylglycerol (Sigma Chemical Co.) and diethyl carbonate (Aldrich Chemical Co.), followed by removal of the benzyl group by hydrogenolysis with palladium on charcoal following the method of Cunningham and Gigg (13). Stearoyl and linoleoyl chloride were obtained from their corresponding acids by reaction with oxaloyl chloride (Aldrich Chemical Co.) by the method of Bosshard et al. (14).

Thin layer chromatography. TLC was carried out on plates of Silica Gel G (250μ) obtained from Analtech (Newark, DE). Visualization of acetal and alk-1-enyl ether products was accomplished by fuchsin-bisulfite spray after exposure of the plates to concentrated hydrochloric acid vapors for one min; aldehydes so liberated were detected as purple to red spots. All products were visualized by spraying the plates with 6% copper sulfate in 50% phosphoric acid, followed by charring.

Gas chromatography. GC was conducted on a Sigma-3 gas chromatograph (Perkin-Elmer, Norwalk, CT) equipped with a capillary injector and flame ionization detector (FID). Separations were obtained using a fused silica wall-coated open tubular column 1.2 m × 0.2 mm i.d. (Hewlett-Packard, Avondale, PA) coated with 0.33μ thick, cross-linked methyl silicone. Determinations were made using helium as a carrier gas (1 ml/min) with a split ratio of 80:1 and the following oven program: seven min at 155 C, then 5 C/min to 200 C. Ethyl heneicosanoate (21:0) served as internal standard because it did not undergo oxidation under the experimental conditions. Signal analysis was accomplished by routing the detector output to the integrating terminal of a Hewlett-Packard 5880A gas chromatograph. A GC trace that shows the separation of the components of interest is shown in Figure 1.

High performance liquid chromatography. HPLC was carried out with a system consisting of a Beckman (Fullerton, CA) Model 110 A solvent delivery module equipped with a Tracor (Austin, TX) Model 945 (FID, an Altex Model 210 loop injector and a 20 μl loop. The HPLC column used was an Altex Ultrasphere-ODS, 5μ particle size, stainless steel column, 4.6 mm i.d. × 25 cm. The FID output was routed to a Hewlett-Packard Model 3390 integrator to determine retention times and peak areas. Triheptadecanoin (NuChek Prep, Elysian, MN) served as internal standard for the HPLC separations shown in Figure 2. Samples were eluted with methylene chloride/

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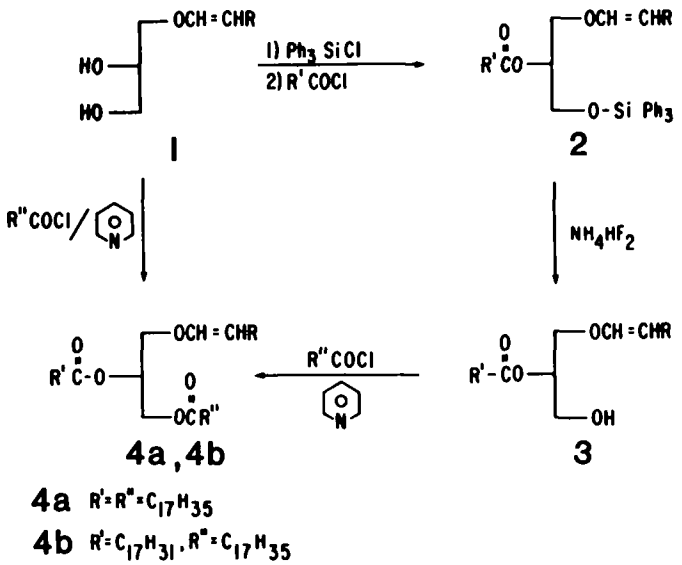
Abbreviations: DMA, dimethyl acetal of hexadecanal; DMSO, dimethylsulfoxide; FID, flame ionization detector; GC, gas chromatography; HPLC, high performance liquid chromatography; ISTD, internal standard; TLC, thin layer chromatography.

acetonitrile (55:45, v/v) at a flow rate of 1 ml/min. Samples were dissolved in the eluent and 20 μl injected.

Synthesis of cis/trans-*1-O-(hexadec-1-enyl)glycerol.* Hexadecanal (20 mmol) was converted to its bis-(2,3-carbonylglycerol) acetal by reaction with glycerol-1,2-carbonate (50 mmol) in the presence of methanesulfonic acid (1 mmol) in 30 ml of benzene/N,N-dimethylformamide (99:1) heated at reflux using a modification of the procedure of Chebyshev et al. (15). The acetal was purified by recrystallization from ether, yield 30%, m.p. 75–79 C. TLC of this product, R_f 0.21, CH_2Cl_2 solvent, showed only one component. Reaction of the acetal with phosphorus pentachloride according to the method of Chebyshev et al. (16) and then elimination of hydrogen chloride with triethylamine, according to the method of Gigg and Gigg (17), gave 1-*O*-(hexadec-1-enyl)glycerol-2,3-carbonate. The latter compound was purified by flash chromatography on silica gel (grade 60, 230–400 mesh, Aldrich Chemical Co.) using ether as eluent, TLC R_f 0.76, ether as eluent. Saponification of 1-*O*-(hexadec-1-enyl)glycerol-2,3-carbonate with methanolic potassium hydroxide (17) gave 1-*O*-(hexadec-1-enyl)glycerol (**1**, Scheme 1) in 68% yield, m.p. 43–44 C recrystallized from isooctane, TLC R_f 0.64 for *trans* isomer, 0.79 for *cis* isomer, eluent ether.

Synthesis of cis/trans-*1-O-(hexadec-1-enyl)-2,3-di-O-stearoylglycerol (4a).* To a solution of 1-*O*-(hexadec-1-enyl)-glycerol 1 (2 mmol) in carbon tetrachloride (10 ml) was added pyridine (10 ml) and stearoyl chloride (4.4 mmol). The reaction mixture was heated at 40 C for one hr under nitrogen, cooled to ambient temperature and filtered through neutral alumina (20 g, 80–200 mesh, Fisher Scientific Co., Fairlawn, NJ) to remove the precipitated pyridinium hydrochloride. The column was washed with an additional 40 ml of carbon tetrachloride, and the eluates were combined. The solvents were removed in vacuo and the residue chromatographed on silica gel (10 g). Elution with methylene chloride/hexane (3:1) gave compound **4a** in 55% yield, R_f 0.90 using methylene chloride/hexane (3:1), m.p. 51–52 C after recrystallization from hexane.



SCHEME 1

Synthesis of cis/trans-*1-O-(hexadec-1-enyl)-2-O-linoleoyl-3-O-stearoylglycerol (4b).* To a solution of 1-(*O*-hexadec-1-enyl)glycerol 1 (2 mmol) in carbon tetrachloride/pyridine (20 ml, 1:1) was added chlorotriphenylsilane (Aldrich Chemical Co.) (2 mmol in 5 ml of carbon tetrachloride). The resulting mixture was stirred at ambient temperature for one hr. TLC of the reaction mixture showed completeness of reaction. Linoleoyl chloride (2 mmol) was added to this solution, and the reaction mixture was heated to 40 C for one hr. The mixture was filtered through a column of neutral alumina (5 g), and the column was washed with chloroform (40 ml). The column eluates were combined, the solvents removed in vacuo, and the residue was chromatographed on silica (20 g) using methylene chloride/hexane (3:1) as eluent. TLC of fractions showed product 2 (Scheme 1) as a mixture of *cis* (R_f 0.62) and *trans* (R_f 0.54) isomers, solvent hexane/ether (4:1), yield 60%. A solution of 2 (1 mmol) in acetone (6 ml) and pyridine (0.2 ml) was heated at reflux for one hr in the presence of ammonium hydrogen fluoride following the procedure of Chebyshev et al. (18). Chromatography on silica gel and elution with methylene chloride/hexane (3:1) gave pure **3**, Scheme 1 as a viscous oil, R_f 0.24, hexane/ether (3:1) in 42% yield. Reaction of **3** (1 mmol) with stearoyl chloride (1.1 mol) in carbon tetrachloride/pyridine (1:1) as described above gave compound **4b**, Scheme 1, in 72% yield after chromatography on silica gel using methylene chloride/hexane (3:1) as eluent. Purified **4b** had an R_f of 0.77, hexane/ethyl acetate (3:1).

Oxidation procedure. Carefully weighed mixtures of either alk-1-enyl ether **4a** or **4b**, the appropriate internal standard and ethyl linoleate in the amounts listed in Table 1 were prepared in 500 μl of pentane. Aliquots of 50 μl then were transferred to each of nine serum vials (120 ml capacity; nominally 100 ml), the pentane removed in vacuo, the vacuum broken with air and the vials sealed with aluminum caps fitted with PTFE-lined butyl rubber septa (Perkin-Elmer). The sealed vials were immersed in a constant temperature water bath at 86 C. Vials were withdrawn at half-hour intervals and allowed to cool to room temperature. Reaction mixtures then were analyzed by either of the following procedures. HPLC analysis: the inside wall of the cooled vial was washed down with 600 μl

TABLE 1

Composition of Reaction Mixtures Prior to Oxidation, in μmol[a]

Component	Run 1[b]	Run 2[b]	Run 3[c]	Run 4[c]	Run 5[c]	Run 6[b]
4a	15.7	14.5	14.4	14.4		
4b					13.5	10.9
18:2[d]		15.2		16.2		
21:0[d,e]	13.3	16.9				13.3
Trihepta-decanoin[e]			14.1	10.6	10.8	

[a]Oxidation carried out under oxygen (ca. 1100 μmol), in sealed vials of 120 ml capacity.
[b]Analysis by GC.
[c]Analysis by HPLC.
[d]As ethyl ester.
[e]Internal standard.

of methylene chloride and 400 μl of acetonitrile, the resulting solution was transferred by means of a Pasteur pipette to a 1-ml serum vial, and 20 μl was injected onto the HPLC column. GC analysis: to each cooled vial 2 ml of 1% H_2SO_4 in methanol was added. The vial was heated on a steam bath for 15 min with occasional shaking and then cooled to room temperature. Saturated aqueous ammonium acetate (4 ml) added, and the resulting mixture was extracted with hexane (2 ml × 2). The combined extracts were dried over anhydrous Na_2SO_4, and 3 μl of the dried extract were injected onto the GC column for analysis.

RESULTS AND DISCUSSION

Synthesis of model neutral plasmalogens. The structures for the model 1-*O*-(alk-1-enyl) glycerides (neutral plasmalogens) synthesized for this study are shown in Scheme 1. Compound **4a**, 1-*O*-(hexadec-1-enyl)-2,3-di-*O*-stearoylglycerol, was prepared directly from 1-*O*-(hexadec-1-enyl)glycerol (**1**) by reaction with two equivalents of stearoyl chloride. Compound **4a** served as a model for studying the 1-*O*-(alk-1-enyl) functionality, found in plasmalogens, for its susceptibility to oxidation. The stearoyl residues of **4a** were not anticipated to be prone to oxidation because we had previously shown that saturated fatty esters, in particular ethyl stearate, are stable under the experimental conditions used in this study (6).

1-*O*-(Hexadec-1-enyl)-2-*O*-linoleoyl-3-*O*-stearoylglycerol, **4b** (Scheme 1), was prepared as a model neutral plasmalogen having internal polyunsaturation. Compound **4b** was obtained from compound **1** by protection of the 3-*O*-position of glycerol with chlorotriphenylsilane to yield compound **2** (Scheme 1). The latter compound, after acylation with linoleoyl chloride and subsequent removal of the silyl protective group, yielded compound **3** (Scheme 1). Reaction of **3** with stearoyl chloride gave the desired compound **4b**. Placement of the linoleoyl residue at the 2-*O*-position of the glycerol backbone of **4b** was opted because this is the position in which unsaturated acyl residues typically are located in naturally occurring neutral and polar plasmalogens (19).

Monitoring techniques for following autoxidation. Table 1 lists the amounts of each substrate used in the oxidation experiments described herein. Two complementary analytical procedures, HPLC and GC, were used in monitoring the oxidative disappearance of the neutral plasmalogens **4a** and **4b**. For Runs 1, 2 and 6, Table 1, substrate depletion was followed by GC using methyl heneicosanoate as an internal standard. In this procedure, the plasmalogens were converted to dimethyacetals and methyl esters with methanol containing 1% sulfuric acid and brief heating at 100 C (Fig. 1). With this procedure, we were able to follow not only the loss of 1-*O*-(hexadec-1-enyl) functionality but also the loss of linoleoyl functionality in **4b**, the latter of which is subject to oxidation under the experimental conditions. Runs 3, 4 and 5, Table 1, were monitored by following the direct disappearance of plasmalogens **4a** and **4b** by HPLC coupled with a FID. Triheptadecanoin was used as internal standard in these experiments. Though loss of linoleoyl functionality in **4b** could not be followed, the loss of added ethyl linoleate to **4a** could be monitored (Fig. 2).

Autoxidation results. The results of the oxidative disappearance of **4a** and **4b** are presented graphically (Figs. 3–6) for a series of experiments carried out at 86 C, a temperature that allowed for a measurable rate of substrate disappearance over a five-hr reaction period. The data are graphed in normalized fashion to show the percentage of remaining components at specified reaction times. All experiments in Table 1 were carried out in sealed vials of sufficient volume (120 ml) to ensure an excess of oxygen (1100 μmol) to eliminate oxygen content as a reaction variable. For oxidation of **4a**, the combined results obtained from Runs 1 and 2 are shown graphically in Figure 3 and combined results from Runs 3 and 4 in Figure 4. For oxidation of **4b**, results from Run 5 are shown in Figure 5 and Run 6 in Figure 6.

Oxidation of 4a. Run 1 (Fig. 3) shows the GC results obtained when compound **4a** (Scheme 1, devoid of polyunsaturation) was subjected to the oxidation conditions employed in these studies. Over the 4.5-hr reaction time of this experiment, a slow but measurable disappearance

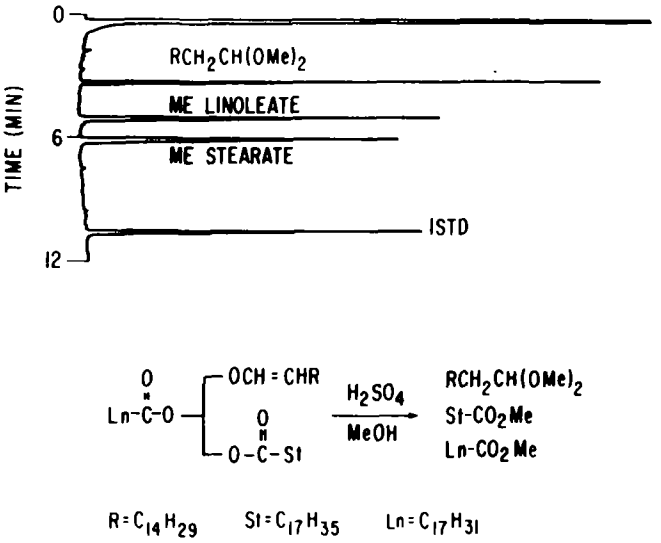


FIG. 1. GC separation of the ethanolysis products of plasmalogen glyceride *4b*; internal standard (ISTD) = methyl heneicosanoate. Conditions as in Materials and Methods.

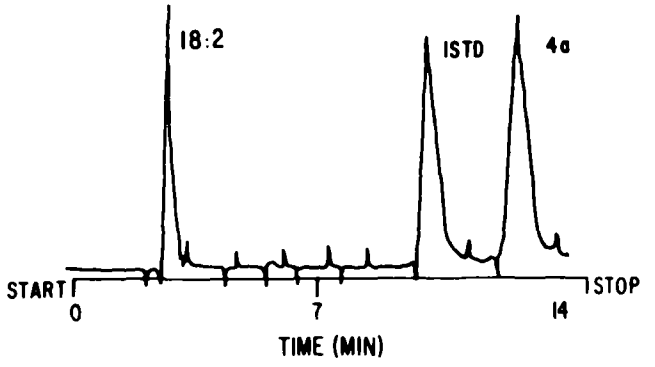


FIG. 2. HPLC of plasmalogen glyceride *4a*, ethyl linoleate (18:2) and triheptadecanoin (internal standard, ISTD). Conditions as in Materials and Methods.

of the 1-*O*-(alk-1-enyl) functionality of 4a was observed; by the end of the reaction period ca. 15% of the original amount of 4a had reacted. This run, which used ethyl heneicosanoate as internal standard, also showed that the stearoyl residues of 4a were unreactive under the conditions employed, a result in agreement with our previous work (6) with ethyl stearate.

The oxidative nature of the disappearance of 4a was established by repeating Run 1 with 0.1% antioxidant, w/w (Tenox 5; Kodak, Rochester, NY). The presence of antioxidant led to complete inhibition of decomposition of 4a over the five-hr reaction time. In addition, reactions failed to proceed when nitrogen was substituted for air in the vial headspace. Thus, loss of alk-1-enyl functionality in 4a cannot be attributed to hydrolysis by trace amounts of acid or water.

Also shown in Figure 3 are the results of Run 2, the oxidation of 4a in the presence of added ethyl linoleate. It is seen that linoleate oxidizes at a much faster rate than does 4a; however, the disappearance of 4a is accelerated in the presence of ethyl linoleate in that by the end of Run 2 almost twice the amount of 4a had reacted compared with Run 1. This result implied that there is accelerated oxidation of the 1-*O*-(alk-1-enyl) functionality of 4a by the oxidation products of linoleate.

In Run 3, Figure 4, the course of the reaction was followed by HPLC, which allowed for the direct measurement of disappearance of 4a, rather than its methanolysis

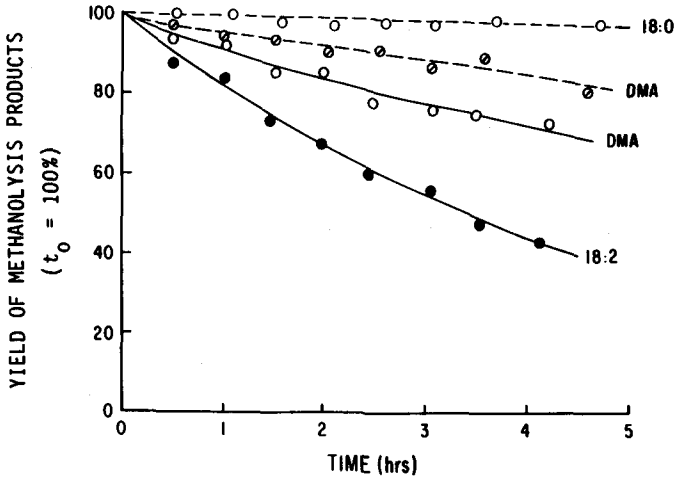


FIG. 3. Course of reaction at 86 C of *4a* as followed by GC of methanolysis products after treatment of oxidized 4a with H_2SO_4/methanol. – – – –, Run 1 (oxidation of *4a* in absence of ethyl linoleate); ——, Run 2 (oxidation of *4a* in presence of ethyl linoleate). 18:0, methyl stearate; DMA, dimethyl acetal of hexadecanal; 18:2, methyl linoleate; internal standard, methyl heneicosanoate; t_o denotes starting time of oxidation reaction.

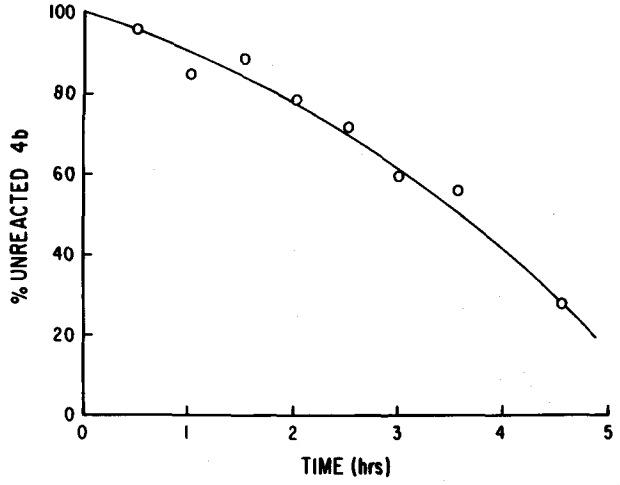


FIG. 5. Course of reaction at 86 C of *4b* (Run 5) as followed by HPLC. Internal standard, triheptadecanoin.

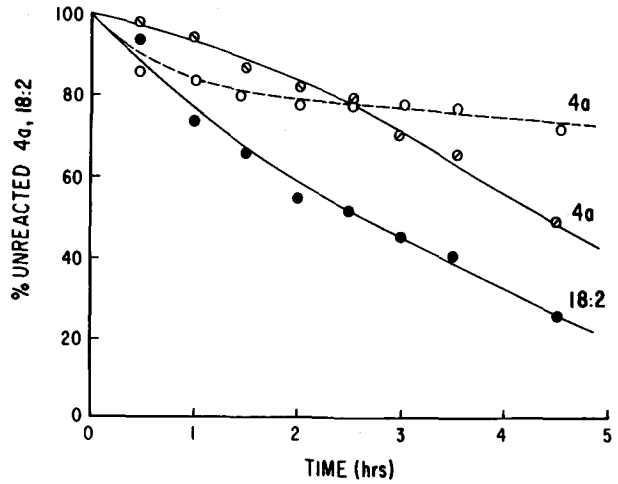


FIG. 4. Course of reaction at 86 C of *4a* as followed by HPLC. – – – –, Run 3 (oxidation of *4a* in absence of ethyl linoleate); ——, Run 4 (oxidation of *4a* in presence of ethyl linoleate). 18:2, ethyl linoleate; internal standard, triheptadecanoin.

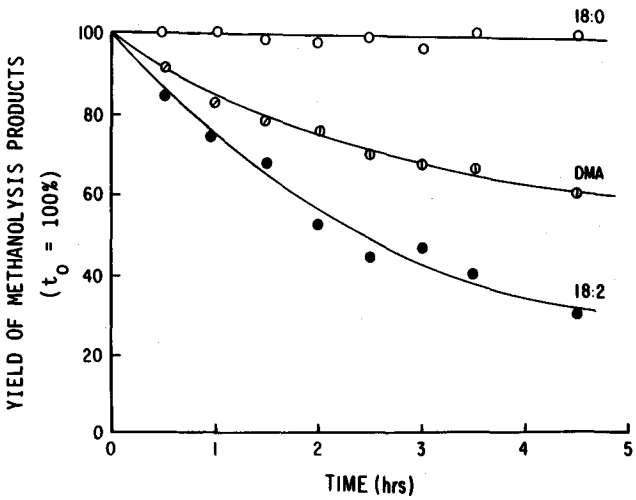


FIG. 6. Course of reaction at 86 C of *4b* (Run 6) as followed by GC of methanolysis products. 18:0, methyl stearate; 18:2, methyl linoleate; DMA, dimethyl acetal of hexadecanal; internal standard, methyl heneicosanoate; t_o denotes starting time of oxidation reaction.

products as in Run 1. In this manner, it was possible to compare the extent of oxidation on each side on the ether structure, that which occurred on the unsaturated (alk-1-enyl) side of the ether vs the saturated (CH_2 of glycerol backbone) side. A comparison of these two runs (1 and 3) shows a 20% disappearance of **4a** in Run 3 vs a 15% loss in Run 1. Accordingly, it is estimated that ca. 75% of the oxidation of **4a** occurs on the unsaturated side of the molecule, and 25% occurs on the saturated side. This result was in agreement with our earlier findings on the oxidation of the simple enol ether model, ethyl hexadec-1-enyl ether.

Run 4, Figure 4, is a repeat of Run 2, oxidation of **4a** in the presence of ethyl linoleate. Run 4 was followed by HPLC instead of by GC. As noted in Run 2, there was an accelerated depletion of **4a** in the presence of added ethyl linoleate; a 35% loss of **4a** was observed in Run 4. The initial rate of loss of **4a** was diminished in the presence of added linoleate, but as linoleate oxidation proceeded the rate of loss of **4a** increased even beyond that of Run 2 (Fig. 3). This result implied that the initial oxidation of linoleate retarded the loss of **4a**; however, as linoleate oxidation proceeded, the accumulation of its oxidation products accelerated the loss of **4a**. This indicated an interaction of linoleate oxidation products, presumably hydroperoxides (20), with the 1-*O*-(alk-1-enyl) function of **4a**. To test this hypothesis, we subjected ethyl linoleate alone to the oxidation conditions and obtained a depletion curve similar to that shown in Figure 4. HPLC of the resulting reaction mixtures showed, in addition to residual ethyl linoleate, a peak eluting earlier in the chromatogram, which could be ascribed to linoleate hydroperoxides (20). Unreacted **4a** in a nitrogen atmosphere then was added to this oxidation mixture, and this mixture was heated at 86 C for one hr. HPLC analysis of the latter mixture showed a loss of ca. 7% of **4a**, indicating a small but significant interaction of the alk-1-enyl function of **4a** with preformed linoleate oxidation products. Presumably, the greater loss of **4a** in the presence of oxidizing linoleate arises from a stronger intermolecular interaction of **4a** with linoleate oxidation intermediates, hydroperoxy radicals, than with linoleate oxidation products, hydroperoxides.

Oxidation of 4b. To assess the effect of intramolecular interaction of linoleate oxidation products with the alk-1-enyl ether functionality of plasmalogens, experiments were carried out using the internally polyunsaturated compound **4b**, Scheme 1, as substrate. Run 5, Figure 5, shows the results obtained when the oxidation of **4b** was followed by HPLC. From this run, it is seen that there is a rapid oxidation of substrate **4b** to the extent that the depletion curve resembles that of linoleate itself. This result was not totally unexpected because compound **4b** contained both of the oxidizable functional groups within its structure. A better picture of the course of oxidation of **4b** was obtained when the reaction was followed by GC, since with this technique it was possible to monitor the loss of alk-1-enyl and linoleoyl functionalities of **4b** separately. As the data show (Run 6, Fig. 6), loss of linoleoyl group amounted to ca. 60%. This confirmed the faster oxidation of linoleoyl residue over the alk-1-enyl functionality. Nevertheless, loss of alk-1-enyl group of **4b** was comparable to or exceeded that observed when **4a** was oxidized in presence of added ethyl linoleate, thus confirming a strong intramolecular interaction between the two oxidizing species. Finally, a comparison of the total oxidation loss of the two functional groups of **4b**, Figure 6, with oxidation loss of intact **4b**, Figure 5, indicated that both functional groups within **4b** are oxidized simultaneously.

From the above series of experiments, it has been demonstrated that the alk-1-enyl functionality of plasmalogens is subject to oxidation under relatively mild oxidizing conditions. More importantly, the data suggest that more easily oxidized polyunsaturated esters accelerate autoxidation of the alk-1-enyl ether functionality of plasmalogens. This acceleration of oxidation by polyunsaturated acyl residues has been shown to occur by either an inter- or intramolecular process. This is highly significant because natural plasmalogens typically also are highly polyunsaturated. Accordingly, the results suggest that oxidation of the alk-1-enyl ether functionality of plasmalogens should not be ignored as a factor that contributes to the oxidative instability of animal tissue or the development of rancidity in meat products.

REFERENCES

1. Davenport, J.B. (1964) *Biochem. J. 90,* 116–122.
2. Sharma, O.P., and Krishna Murti, C.R. (1980) *J. Sci. Ind. Res. 39,* 110–117.
3. Barclay, L.R.C., and Ingold, K.U. (1981) *J. Am. Chem. Soc. 103,* 478–6485.
4. Mottram, D.S., and Edwards, R.A. (1983) *J. Sci. Food Agric. 34,* 517–522.
5. Mowri, H., Nojima, S., and Inoue, K. (1984) *J. Biochem. (Tokyo) 95,* 551–558.
6. Marmer, W.N., Nungesser, E., and Foglia, T.A. (1986) *Lipids 21,* 648–651.
7. Yavin, E., and Gatt, S. (1972) *Eur. J. Biochem. 25,* 431–436.
8. Yavin, E., and Gatt, S. (1972) *Eur. J. Biochem. 25,* 437–446.
9. Barker, M.O., and Brin, M. (1975) *Arch. Biochem. Biophys. 166,* 32–40.
10. Gorman, A.A., Gould, I.R., and Hamblett, I. (1982) *J. Am. Chem. Soc. 104,* 7098–7104.
11. Jefford, C.W., Kohmoto, S., Boukouvalas, J., and Burger, U. (1983) *J. Am. Chem. Soc. 105,* 6498–6499.
12. Fenselau, A.H., and Moffatt, J.G. (1966) *J. Am. Chem. Soc. 88,* 1762–1765.
13. Cunningham, J., and Gigg, R. (1965) *J. Chem. Soc. Pt. 1,* 1553–1554.
14. Bosshard, H.H., Morg, R., Schmid, M., and Zuellinger, H. (1950) *Helv. Chim. Acta 42,* 1653–1658.
15. Chebyshev, A.V., Serebrennikova, G.A., and Evstigneeva, R.P. (1977) *J. Org. Chem. (USSR) 13,* 644–648.
16. Chebyshev, A.V., Serebrennikova, G.A., and Evstigneeva, R.P. (1979) *Sov. J. Bioorg. Chem. 4,* 469–473.
17. Gigg, J., and Gigg, R. (1968) *J. Chem. Soc. C,* 16–21.
18. Chebyshev, A.V., Serebrennikova, G.A., and Evstigneeva, R.P. (1978) *Sov. J. Bioorg. Chem. 3,* 997–1003.
19. Curstedt, T. (1983) in *Ether Lipids* (Mangold, H.K., and Paltauf, F., eds.) pp. 1–16, Academic Press, New York.
20. Koskas, J.P., Cillard, J., and Cillard, P. (1983) *J. Chromatogr. 258,* 280–283.

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Lipids of Dermatophytes. III. Sterol-induced Changes in the Lipid Composition and Functional Properties of *Epidermophyton floccosum*

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Sterol supplementation, alone or in the presence of cerulenin, resulted in an increase in the total sterol content of *Epidermophyton floccosum*. While the total phospholipid levels of *E. floccosum* exhibited only marginal changes with sterol supplementation, the fatty acid profiles of these phospholipids were highly varied. In the presence or absence of cerulenin, the oleic acid content of phospholipids were increased significantly by cholesterol supplementation, whereas linoleic acid levels were enhanced by ergosterol supplementation. These variations resulted in higher unsaturated/saturated phospholipid fatty acid ratios in sterol-supplemented cells. The uptake of labeled amino acids (aspartic acid, lysine, glycine) was influenced by sterol supplementation. Alterations in the number of binding sites for the membrane probe, 1-anilino-naphthalene-8-sulfonate (ANS), were seen based on Scatchard plot calculations. The results indicate a correlation between sterol-induced changes in membrane lipid composition and function.
Lipids 23, 435–438 (1988).

Sterols, the important components of some prokaryotes and eukaryotes, function as major determinants of fluidity, integrity and membrane structure (1,2). Yeast cells are known to exhibit altered sterol contents when grown in media supplemented with ascorbic acid, hydroquinone, methylene blue or potassium persulfate, resulting in altered amino acid uptake (3,4). In dermatophytes, little is known about the dependency of cellular functions on sterol composition. To explore these aspects in human pathogenic fungi, *Epidermophyton floccosum* cultures were grown in media supplemented with cholesterol or ergosterol in presence/absence of cerulenin, a known inhibitor of fatty acid and sterol synthesis. Further, attempts have been made to correlate sterol-induced alterations in the lipid composition with the structural and functional changes in the membranes of *E. floccosum*.

MATERIALS AND METHODS

Materials. Cholesterol, ergosterol, glycine, aspartic acid and lysine were procured from Sigma Chemical Co. (St. Louis, MO). Cerulenin was obtained from Makor Chemicals (Jerusalem, Israel). Labeled amino acids [U-^{14}C]lysine (sp. act. 288 mCi/mmol); [2-^{14}C]glycine (sp. act. 11.2 mCi/mmol), [U-^{14}C]aspartic acid (sp. act. 152 mCi/mmol) were obtained from BARC, Bombay. Novozyme '234' was purchased from M/S Novo Industries (Bagsvaerd, Denmark), and Cellulase CP was procured from Sturge Biochemicals (Selby, U.K.). Ficoll-paque was procured from Pharmacia Fine Chemicals Co. (Uppsala, Sweden). 1-Anilinonaphthalene-8-sulfonate (Mg salt) was obtained from Fluka (Buchs, Switzerland). Membrane filters (0.45 μM) and a 12-place sampling manifold, used for the amino acid uptake experiments, were obtained from Whatman Inc., London, and Millipore Inc. (Bedford, MA), respectively. All other chemicals used were of the highest quality available.

Growth of culture. Source and maintenance of *E. floccosum* have been described (5). *E. floccosum* was grown in Sabouraud's broth as shaking cultures. Minimum inhibitory concentration of cerulenin and the optimum concentrations of sterols (dissolved in minimum amounts of ethanol) were added directly to the sterile medium before inoculation. Sterol-grown cells, in the presence/absence of cerulenin, were harvested in mid-log phase (5 days) and then processed for sterols, DNA, lipid extraction and membrane studies.

Quantitation of phospholipids. The lipids were extracted from the cells by Folch's extraction procedure (6). Total phospholipids were estimated by the method of Bartlett as modified by Marinetti (7). Single-dimensional thin layer chromatography was carried out to resolve individual phospholipids, using the solvent system chloroform/methanol/7 N ammonia (65:25:4, v/v). Total phospholipids were separated from neutral lipids by acetone precipitation and were used for fatty acid analysis. The fatty acids were converted into their methyl esters by transesterification with methanol in the presence of thionyl chloride, as described (5). The methyl esters were analyzed on a M/S Nucon Engineers, AIMIL Gas Chromatograph, fitted with a flame ionization detector, on columns containing 20% diethylene glycol succinate (DEGS) on 60–80 mesh chromosorb-W. Nitrogen, at a flow rate of 40 ml/min, was used as the carrier gas. The fatty acids were quantitated by triangulation.

Quantitation of sterols. Sterols were extracted by boiling the cells in 10% alcoholic KOH for one hr. The hydrolysates were cooled and extracted three times with petroleum ether. Sterols were estimated by the method of Zlatkis et al. (8).

Quantitation of DNA. DNA extraction was achieved by homogenizing the cells in 10% cold trichloroacetic acid. The pellet obtained after centrifugation at 2,000 × g was dissolved in a known volume of 0.5 M perchloric acid and digested at 80 C in water bath for 15 min. The samples again were centrifuged, and supernatant was taken for DNA estimation by the method of Burton (9).

Preparation of spheroplasts. Spheroplasts were prepared by the procedure of Larroya et al. (10). Log-phase cells (1 g) were incubated under sterile conditions with 30 mg each of two commercial cell-wall degrading enzymes, novozyme '234' and cellulase CP, in a conical flask in citrate phosphate buffer (pH 6.5), containing 0.7 M NaCl, for 10 hr in a shaker at 27 C. Formation of spheroplasts was monitored microscopically. At the end of incubation, the reaction mixture was removed by centrifugation at 1,000 × g for 10 min. The pellet was washed twice with citrate phosphate buffer and lightly homogenized. Spheroplast preparation then was purified by centrifugation at 400 × g for 15 min on a ficoll-paque density gradient. The purified spheroplast preparation was used

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Abbreviations: ANS, 1-anilinonaphthalene 8-sulfonate; LPC, lysophosphatidylcholine; MIC, minimum inhibitory concentration; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TPL, total phospholipid.

for structural studies with the fluorescent probe 1-anilinonaphthalene-8-sulfonate (ANS).

Amino acid uptake studies. Amino acid uptake in whole cells was determined by incubating 1 ml of cell suspension (40–50 mg fresh wt. of cells in 1 ml of citrate phosphate buffer, pH 6.5) at 27 C for seven min, which was within the linear range of uptake. The reaction was initiated with the addition of amino acid (final concentration was 4 mM), and it was terminated by adding chilled buffer and filtered rapidly through 0.45 μM membrane filters. The cells on the filter paper were washed twice, dried and weighed. Radioactivity on the filters was counted in a toluene-based scintillation fluid in a Packard Tricarb Liquid Scintillation Counter.

Structural studies with ANS. 1-Anilinonaphthalene 8-sulfonate (ANS) was used to study the structural changes in the membranes of spheroplasts prepared from variously grown *E. floccosum* cells. The basic assay mixture consisted of a total volume of 2 ml in 10 mM citrate phosphate buffer, pH 6.5, containing 0.7 M NaCl, ANS conc. 10–100 μM and spheroplast protein ranging from 20 to 200 μg. The fluorescence emission was recorded in a Kontron SFM-25 Spectrofluorimeter. The number of binding sites were calculated from the Scatchard plot (11). Protein was estimated by Lowry's method (12).

RESULTS AND DISCUSSION

Manipulation of membrane lipid composition by supplementing various additives to the growth medium is a valuable tool for studying the role of various lipid components in microorganisms. To examine the role of sterols, *E. floccosum* was grown in the presence of optimum concentration of ergosterol (natural sterol) or cholesterol (a sterol foreign to the fungus), alone or in combination with the minimum inhibitory concentration (MIC) of cerulenin (0.5 μg/ml). Cerulenin inhibits more than 90% of growth and endogenous fatty acid synthesis, and partially blocks (60%) phospholipid and sterol synthesis at the MIC (13). Different concentrations of cholesterol and ergosterol ranging from 0 to 30 μg/ml were supplemented in the medium, and no inhibition in the growth as compared to control was observed up to 10 μg/ml. This concentration of sterol(s) was used throughout this study.

Ergosterol supplementation, with or without cerulenin, did not alter the total phospholipid (TPL) levels as compared with the control. However, the total phospholipid levels were significantly decreased in cells supplemented with cholesterol alone or along with cerulenin (Table 1). Individual phospholipid composition also was examined. Phosphatidylserine (PS) and phosphatidylinositol (PI) were pooled together, the latter being the minor component with a mobility close to PS. Minor changes were observed in all the phospholipid components, with a significant decrease in PS + PI and phosphatidylcholine (PC) levels in cholesterol-supplemented cells. Ergosterol supplementation in the presence or absence of cerulenin resulted in a significant increase in choline-containing lipids, which probably is due to enhanced methylation of phosphatidylethanolamine (PE) to PC. In yeast mutant

TABLE 1

Effect of Supplementation of Sterols in the Presence and Absence of Cerulenin on the Lipid Composition of *E. floccosum*

mg/g Dry wt. of cells	Control	Cholesterol	Ergosterol	Cerulenin + cholesterol	Cerulenin + ergosterol
TPL	13.95 ± 1.01 (3.04 ± 0.39)	9.54 ± 1.5* (2.35 ± 0.13)*	15.57 ± 1.12[a] (3.03 ± 0.38)[a]	11.25 ± 1.06* (2.80 ± 0.55)[a]	13.15 ± 1.55[a] (2.76 ± 0.37)[a]
LPC	0.99 ± 0.43	0.70 ± 0.14[a]	2.25 ± 0.31*	1.45 ± 0.23[a]	2.39 ± 0.12**
PS + PI	3.92 ± 0.44	2.54 ± 0.34*	4.12 ± 0.47[a]	2.17 ± 0.22**	3.22 ± 0.30[a]
PC	3.91 ± 0.58	2.48 ± 0.31*	3.98 ± 0.29[a]	3.42 ± 0.40[a]	3.56 ± 0.56[a]
PE	2.07 ± 0.23	1.39 ± 0.39[a]	1.85 ± 0.47[a]	2.11 ± 0.10[a]	1.81 ± 0.38[a]
CL	1.30 ± 0.22	1.03 ± 0.18[a]	0.88 ± 0.29[a]	1.45 ± 0.20[a]	1.27 ± 0.22[a]
UK	1.67 ± 0.19	1.22 ± 0.24[a]	2.74 ± 0.12**	0.59 ± 0.12**	0.80 ± 0.12**
$\frac{LPC + PC + PE}{PS + PI}$	1.77	1.79	1.96	3.21	2.40
Sterols	8.04 ± 0.91 (1.85 ± 0.39)	10.26 ± 0.57* (2.58 ± 0.35)*	10.13 ± 0.57* (2.13 ± 0.33)[a]	14.25 ± 1.59** (3.40 ± 0.13)**	10.88 ± 1.88[a] (2.26 ± 0.10)[a]
Sterol/TPL	0.57	1.07	0.65	1.26	0.82
$\frac{PC + LPC}{PE}$	2.31	2.28	3.36	2.30	3.28

[a]Nonsignificant.

The values are mean ±SD of three independent batches analyzed in duplicate. *, $p \leq 0.05$; **, $p \leq 0.01$; statistical significance of the observed difference between the means of test and control, was determined by applying Student's t-test. Values in parentheses indicate the data expressed on mg/mg DNA basis.

TPL, total phospholipid; PI, lysophosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; UK, unknown phospholipid.

Concentrations used: cholesterol, 10 μg/ml; ergosterol, 10 μg/ml; cerulenin, 0.5 μg/ml.

GL7, ergosterol also has been shown to stimulate the methlation of PE to PC (14). The increased levels of lipophosphatidylcholine (LPC) in cells supplemented with ergosterol (both in presence and absence of cerulenin) might be due to the conversion of PC to LPC because dermatophytes possess an active phospholipase A (15). Alterations in the individual phospholipid composition resulted in changes in the ratio of zwitterionic to anionic phospholipids and the ratio of choline-containing phospholipids to PE in the variously grown cells (Table 1). The levels of total sterols (mainly free sterols because esterified sterols represent less than 5% of the total sterols) were increased on supplementation of cholesterol and ergosterol, alone or in the presence of cerulenin. These results indirectly indicate the capacity of the cells to take up exogenous sterols. Sterol to phospholipid ratio increased in all the supplementations as compared with control, in the order, ergosterol (1.1-fold) < cerulenin plus ergosterol (1.4-fold) < cholesterol (1.8-fold) < cerulenin + cholesterol (2.2-fold) (Table 1).

Sterol supplementation in the presence or absence of cerulenin not only affected the phospholipid and sterol composition but also the acyl groups of phospholipids. A significant decrease in the levels of palmitic acid were observed in all the types of cells as compared with control (Table 2). Cholesterol supplementation in the presence and absence of cerulenin resulted in a significant increase in oleic acid levels, whereas linoleic acid levels increased significantly on supplementation of ergosterol with/without cerulenin. The observed increase in oleic acid content of cholesterol-grown *E. floccosum* cells is similar to that observed in yeast sterol auxotroph (RD5-R) (16). Supplementation of ergosterol, cerulenin plus ergosterol/cholesterol resulted in a significant increase in U/S ratio, whereas supplementation of cholesterol alone did not alter the U/S ratio significantly. In yeast, increased percentages of saturated fatty acids were observed on supplementation of the growth medium with sterols (17). It has been further suggested that sterol replacement regulates the fatty acid composition to a considerable degree.

The lipid composition of the cell membrane plays an important role in regulating the functions of the cells. Therefore, the functional properties of membranes in response to changes in lipid composition, as a result of sterol supplementation, were studied by measuring the steady-state accumulation of amino acids (one each of basic, neutral and acidic amino acids). It is evident from Table 3 that amino acid uptake was altered in the variously grown cells. The changes may be attributed to alterations observed in phospholipid composition including those in the ratio of zwitterionic and anionic lipid species as well as degree of unsaturation of acyl groups of phospholipids, as has been demonstrated earlier in dermatophytes (18,19), bacteria (20) and yeast (21). These changes also could be due to a change in the affinity of the carriers of these amino acids. Singh et al. (22) have

TABLE 2

Fatty Acid Composition of Phospholipids of *E. floccosum* Cultures Grown in the Presence of Sterols, With or Without Cerulenin

Supplement	Relative percentage of phospholipid fatty acids					U/S fatty acids ratio
	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	
Nil (control)	t	41.96 ± 1.87	13.26 ± 1.79	20.40 ± 1.51	24.36 ± 2.34	0.81 ± 0.12
Cholesterol	t	34.63 ± 2.04**	13.38 ± 0.91[a]	36.00 ± 2.06***	15.98 ± 2.45*	1.08 ± 0.15[a]
Ergosterol	t	29.01 ± 2.70**	15.86 ± 1.83[a]	15.53 ± 1.33*	39.90 ± 1.94***	1.23 ± 0.13**
Cerulenin and cholesterol	t	32.85 ± 3.60*	8.34 ± 2.28*	40.03 ± 4.64**	18.72 ± 1.94[a]	1.43 ± 0.19**
Cerulenin and ergosterol	t	22.60 ± 2.01***	8.10 ± 1.29*	14.24 ± 2.11*	55.04 ± 1.96***	2.26 ± 0.19***

[a]Nonsignificant.

Values are mean ±SD of three independent batches.

*, $p \leq 0.05$; **, $p < 0.01$; ***, $p \leq 0.001$.

Statistical significance of the observed difference between the means of test and control was determined by applying Student's t-test.

t, Trace amounts; S, saturated fatty acids; U, unsaturated fatty acids.

TABLE 3

Amino Acid Uptake in Variously Grown Cells of *E. floccosum*

Supplement	Lysine[a]	Aspartic acid[a]	Glycine[a]
Nil	5.25 ± 0.46	3.23 ± 0.28	4.08 ± 0.32
Cholesterol	4.80 ± 0.34[b]	4.08 ± 0.51[b]	5.34 ± 0.48*
Ergosterol	3.81 ± 0.35*	2.83 ± 0.19[b]	3.55 ± 0.17[b]
Cerulenin + cholesterol	8.67 ± 0.93**	2.71 ± 0.20[b]	3.38 ± 0.14*
Cerulenin + ergosterol	5.99 ± 0.42[b]	2.43 ± 0.17*	7.71 ± 0.39***

[a]Results expressed in nmol/mg dry wt./seven min. Values are mean ±SD of three independent batches.

[b]Nonsignificant.

*p, ≤ 0.05; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Statistical significance of the observed difference between the means of test and control, was determined by applying Student's t-test.

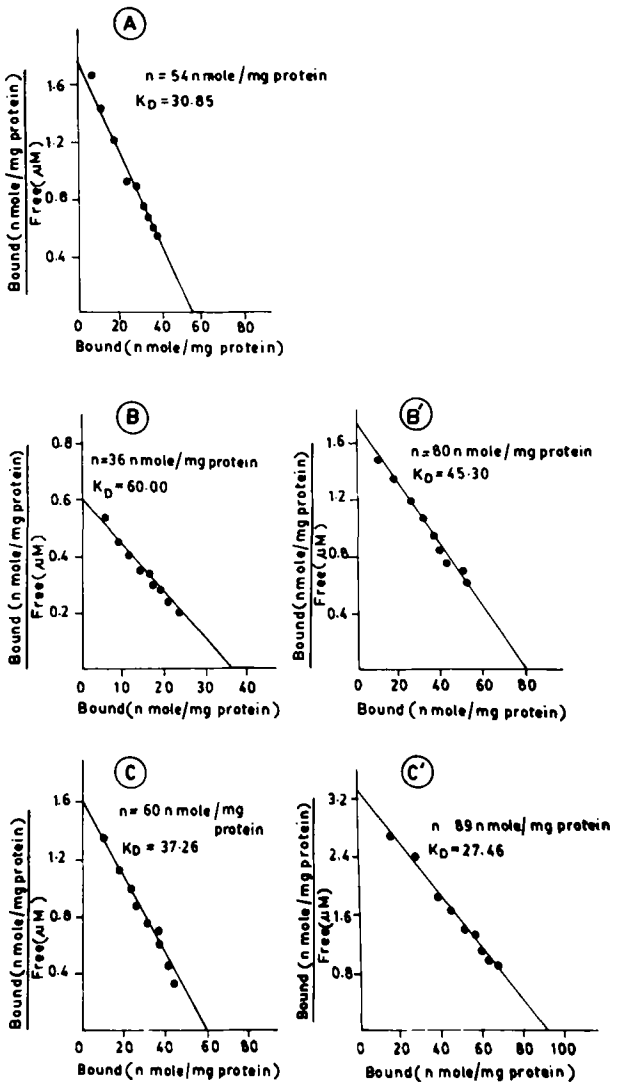

FIG. 1. Scatchard plots of ANS binding (based on one batch run in duplicate) with *Epidermophyton floccosum* spheroplasts. A, spheroplasts of control cells; B, spheroplasts of cholesterol-grown cells; B', spheroplasts of cholesterol plus cerulenin-grown cells; C, spheroplasts of ergosterol-grown cells; C', spheroplasts of ergosterol plus cerulenin-grown cells.

demonstrated alterations in the uptake of various amino acids due to changes in the ergosterol levels in *C. albicans*, similar to our observations with *E. floccosum*.

Structural alterations due to changes in the membrane surface charge density, in the spheroplast membrane prepared from *E. floccosum* cultures grown in sterol-supplemented media, were monitored using an ANS membrane probe. ANS fluorescence provides information about membrane surface charge and membrane potential (23, 24). The Scatchard plot analysis showed decreased binding sites in spheroplasts prepared from cholesterol-supplemented cells, whereas no alteration was observed in ergosterol-supplemented cells (Fig. 1). The number of binding sites were found to increase in spheroplasts prepared from cells grown in the presence of cerulenin and cholesterol/ergosterol, as compared with the control cells. Increased/decreased binding sites might be due to changes in the membrane surface charge resulting from changed ratio of zwitterionic to anionic phospholipids as well as altered ratio of unsaturated to saturated fatty acids, as seen from Tables 1 and 2. In addition, PC also is known to regulate the charge of the membranes (25), and a significant decrease in PC content was seen in cholesterol-supplemented cells, which possibly explains the decreased binding sites in spheroplasts prepared from these cells. The changes observed in ANS binding also may be due to changed hydrophobic environment around the dye due to integral membrane proteins (25). The dye mainly binds to the membrane surface, hence changes observed demonstrate a role of membrane phospholipids, sterols and proteins, because these are the only components of the cell that come in contact with ANS.

We have shown that supplementation of sterols, alone or in the presence of cerulenin, induces changes in the lipid composition of *E. floccosum*, which in turn affects its structural and functional properties.

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REFERENCES

1. Shimokawa, O., Kato, Y., and Nakayama, H. (1986) *J. Med. Vet. Mycol. 24*, 327-336.
2. Borochov, H., Abbott, R.E., Schachter, D., and Shinitzky, M. (1979) *Biochemistry 18*, 251-255.
3. Khanna, M., and Shukla, O.P. (1976) *Ind. J. Exp. Biol. 14*, 729-730.
4. Singh, M., Jayakumar, A., and Prasad, R. (1979) *Microbios 24*, 7-17.
5. Khuller, G.K., Chopra, A., Bansal, V.S., and Masih, R. (1981) *Lipids 16*, 20-22.
6. Folch, J., Lees, M., and Sloane-Stanley, G.H.S. (1957) *J. Biol. Chem. 226*, 497-509.
7. Marinetti, G.V. (1962) *J. Lipid Res. 3*, 1-11.
8. Zlatkis, A., Zak, B., and Boyles, A.J. (1953) *J. Lab. Clin. Med. 41*, 486-492.
9. Burton, K. (1956) *Biochem. J. 62*, 315-318.
10. Larroya, S., Johl, P.P., Pancholi, V., and Khuller, G.K. (1984) *IRCS Med. Sci. 12*, 1064-1065.
11. Azzi, A. (1975) *Q. Rev. Biophys. 8*, 237-316.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265-275.
13. Sanadi, S., Pandey, R., and Khuller, G.K. (1987) *Biochim. Biophys. Acta 921*, 341-346.
14. Kawasaki, S., Ramgopal, M., Chin, J., and Bloch, K. (1985) *Proc. Natl. Acad. Sci. USA 82*, 5715-5719.
15. Chopra, A., and Khuller, G.K. (1984) *IRCS Med. Sci. 12*, 179-180.
16. Bottema, C.D.K., Rodriguez, R.J., and Parks, L.W. (1985) *Biochim. Biophys. Acta 813*, 313-320.
17. Bloch, K.E. (1983) *CRC Crit. Rev. Biochem. 14*, 47-92.
18. Larroya, S., and Khuller, G.K. (1985) *Lipids 20*, 11-15.
19. Larroya, S., and Khuller, G.K. (1986) *Ind. J. Biochem. Biophys. 23*, 9-12.
20. Ohta, T., Okuda, S., and Takahashi, H. (1977) *Biochim. Biophys. Acta 466*, 44-56.
21. Trivedi, A., Singhal, G.S., and Prasad, R. (1983) *Biochim. Biophys. Acta 729*, 85-89.
22. Singh, M., Jayakumar, R., and Prasad, R. (1979) *Biochim. Biophys. Acta 555*, 42-55.
23. Azzi, A. (1969) *Biochem. Biophys. Res. Commun. 37*, 254-260.
24. Brocklehurst, J.R., Freedman, R.P., Hancock, D.J., and Radda, G.K. (1978) *Biochem. J. 116*, 721-731.
25. Slavik, J. (1982) *Biochim. Biophys. Acta 694*, 1-25.

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Occurrence of Cholesterol as a Major Sterol Component in Leaf Surface Lipids

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Cholesterol has been detected as one of the major sterols in the surface lipids of higher plant leaves. It was widely distributed among the plant leaves of various species as a common main sterol component with a few exceptions. The content of cholesterol amounted to 71.5% of the total sterols in the surface lipids of rape leaves. However, the proportion of cholesterol in the intracellular lipids of rape leaves was lower than that in the surface lipids, and the seed lipids contained only a trace amount of cholesterol, as reported in the literature. In the leaf surface lipids examined, a minor amount of cholestanol associated with cholesterol often was detected by capillary gas chromatography and gas chromatography-mass spectrometry. The related analysis for the surface lipids of fruits showed that cholesterol was one of the major component sterols also in those lipids of several species.
Lipids 23, 439-444 (1988).

Cholesterol up to now has been believed to be a minor component sterol, 4-desmethylsterol, in higher plants. It was detected in vegetable oils by gas chromatography and existed in a small proportion up to 5% of the total sterols or mostly in only trace amounts (1-3). Previous workers have reported that, in general, a slight amount of cholesterol or no cholesterol was found in the plant seed lipids of various species (4-7,11,14,15). The component sterol data for roots (6,8), leaves (7-13), pericarps (8,12) and seedlings (6,8,11) also showed that cholesterol was a minor component sterol in their sterol fractions. However, several studies have revealed the existence of cholesterol as a major component sterol in chloroplasts and other subcellular organelles of leaves (16,17) and shoots (18), poinsettia plant (19), pollens (20) and *Sorghum bicolor* grains in their early developing stage (14).

In analyzing the leaf and silique waxes of rape, we have found that they contained an unusually large proportion of cholesterol in their sterol fractions. Our further experiments for the detection of cholesterol also have showed that cholesterol was present as one of the usual major component sterols in the leaf surface lipids of various plant species, though several species had cholesterol as the minor component sterol. Moreover, a substantial proportion of cholesterol has been observed in the component sterols of several fruit surface lipids. This paper describes the detection and determination of cholesterol in plant lipids by capillary gas chromatography and its identification by gas chromatography-mass spectrometry (GC-MS).

MATERIALS AND METHODS

Plant materials. Fully matured seeds and leaves of rape *(Brassica napus)* and the immature siliques of rape harvested at 30 days after flowering were used for these experiments. The full-grown leaves of the cultivated *Viburnum awabuki* tree were collected in the summertime. Fresh leaves of spinach *(Spinacia oleracea),* cabbage *(Brassica oleracea),* radish *(Raphanus sativus),* crown daisy *(Chrysanthemum coronarium)* and leek *(Allium tuberosum),* all sold commercially as food, were used.

Immature green fruits, such as the legumes of broad bean *(Vicia faba),* the capsules of okra *(Abelmoschus esculentus)* and the fruits of green pepper *(Capsicum annuum)* were obtained from vegetable markets.

Reference sterols and solvents. Cholesterol, campesterol, stigmasterol and sitosterol were obtained from Sigma Chemical Co. (St. Louis, MO). Cholestanol was purchased from the Nakarai Chemical Co. (Kyoto, Japan). Brassicasterol was prepared from rapeseeds. All solvents used for extraction were purified by distillation before use.

Isolation of sterols. Leaves and fruits (100-1000 g) without cutting were immersed in hexane (1-3 l) and allowed to stand for two days at room temperature with occasional shaking. The hexane extract was separated by filtration, and the residual plant bodies were then reextracted twice by gentle refluxing with fresh hexane for each one hr. All hexane extracts were combined, and the separated water layer was removed. After drying over anhydrous sodium sulfate, the hexane layer was evaporated to dryness under reduced pressure. The extracted lipids contained mainly the plant surface lipids. In the case of rape leaves, a portion of the extracted lipids was divided for the separation of the steryl lipid classes.

The lipids were hydrolyzed with a solution of potassium hydroxide (1 g) in aqueous ethanol (5 ml) by refluxing for two hr. The hydrolysates were extracted with diethyl ether, and the ether extract was washed with water and dried with anhydrous sodium sulfate. Evaporation of ether from the extract gave unsaponifiable matter.

The component lipid classes in the unsaponifiable matter were separated on Silica Gel G plates, with hexane/diethyl ether/acetic acid (70:30:1, v/v/v) used as the developing solvent. The band corresponding to that of free sterol was detected by Rhodamine 6G spraying, scraped and then extracted with ether and hexane. The extracted lipid fraction was comprised of a mixture of the sterols originated mainly from free sterols and steryl esters in the surface lipids.

Intracellular lipids were extracted by homogenization with chloroform/methanol (1:1, v/v) from the plant residue after the hexane extraction described above. The extracted rape leaf lipids were separated into four lipid classes containing sterols. Thin layer chromatography (TLC) was applied on Silica Gel G plates for the separation of free sterols from steryl esters with the solvent system, hexane/diethyl ether/acetic acid (70:30:1, v/v/v), and of sterylglycosides from acylsterylglycosides with chloroform/methanol/acetone (80:16:4, v/v/v). The component sterols were recovered separately from sterylglycosides and acylsterylglycosides by the acid hydrolysis

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Abbreviations: GC-MS, gas chromatography-mass spectrometry; RRT, relative retention time; TLC, thin layer chromatography; TMS, trimethysilyl.

with 0.5 N HCl in methanol for two hr, followed by the alkali hydrolysis described above in the case of acylsterylglycosides.

Reversed-phase TLC using Kieselguhr G plates impregnated with kerosene was employed for purification of cholesterol with the developing solvent of acetic acid/acetonitrile (1:2, v/v).

Gas chromatography (GC). Sterols were analyzed by packed-column and capillary GC as free sterols or trimethylsilyl ethers of sterols (TMS sterols). TMS sterols were prepared by the reaction of sterols with N,O-bis-(trimethylsilyl)acetamide (BSA)/pyridine (1:3, v/v) at 80 C for 10 min.

The free sterols and their TMS derivatives were separated and identified on a glass column (3 mm × 2 m) packed with 3% SE-30 on Chromosorb W at 260 C.

Capillary GC was carried out for precise analysis of sterol with a Shimadzu GC-8A gas chromatograph equipped with a flame-ionization detector. A fused silica capillary column (chemically bonded type, 0.24 mm i.d. × 50 m), ULBON HR-52, which corresponded to SE-52 (Shinwakako Co., Kyoto, Japan), was used. The column temperature was 280 C, and the carrier gas was nitrogen at a flow rate of 0.7 ml/min and split ratio 1/136. Relative retention time (RRT) to TMS cholesterol (retention time, 11.5–12 min) was employed for characterization of the TMS sterols.

Gas chromatography-mass spectrometry. Electron impact GC-MS data were obtained by using a model JEOL JMS-HX 100 system fitted with a glass column (2 mm × 1 m) packed with 1% OV-1 on Chromosorb W, with a column temperature of 245 C; ion source temperature was 200 C, accelerating voltage 5.0 kV, and ionizing voltage 70 eV.

Another model Hitachi M-80A system for GC-MS also was employed. In this system, the GC separation was carried out at 260 C on a column (3 mm × 1 m) packed with 2% OV-101 on Chromosorb WHP. The ionizing voltage was 20 eV, and the ion source temperature was 180 C.

Other analytical methods. ^{1}H NMR spectroscopy was performed at 60 MHz on a Hitachi instrument, model R-600, in $CDCl_3$ with tetramethylsilane as internal standard. Addition of increasing amounts of the shift reagent, $Eu(fod)_3$, made it possible to assign some of the proton signals on the NMR spectra of sterols (21). The infrared (IR) spectrum of sterol was obtained with a KBr disk.

Sterols were estimated by the color reaction with acetic anhydride/concentrated sulfuric acid (19:1, v/v). Intensities of the developed color were measured at 620 nm after 30 min.

RESULTS

Separation of rape sterols by capillary GC. A mixture of sterols was recovered from leaves, seeds or fruits by hexane extraction, saponification and subsequent TLC separation. The sterol content of surface lipids ranged from 3 to 50 mg per 100 g of the individual fresh plant matter examined.

Capillary GC showed good separations for TMS derivatives of sterols on a fused silica capillary column, ULBON HR-52 (Fig. 1). Separation and identification were possible for cholesterol and cholestanol in their mixtures that were inseparable on SE-30, OV-1 and OV-101 packed

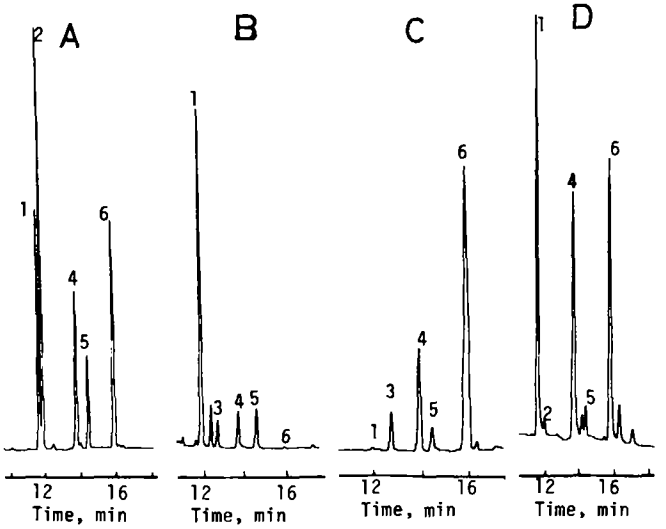

FIG. 1. Capillary gas chromatograms of TMS sterols from plant lipids. A, standard sterols; B, sterols from surface lipids of rape leaves; C, sterols from intracellular lipids of rape seeds; D, sterols from surface lipids of broad bean *(Vicia faba)* fruits. Peaks of TMS sterols: 1, cholesterol; 2, cholestanol; 3, brassicasterol; 4, campesterol; 5, stigmasterol; 6, sitosterol; other peaks were not identified. Column, ULBON HR-52 (0.24 mm i.d. × 50 m); temperature, 280 C.

columns. A marked difference in sterol composition was observed between rape leaf and seed lipids. The main sterol component of rape leaf surface lipids was cholesterol (71.5%), and a minor sterol was sitosterol (0.6%), whereas the seed intracellular lipids contained sitosterol as a dominant sterol (67.3%) and a trace amount of cholesterol (0.7%) as shown in Figure 1 and Table 1 (1–3). Cholesterol was a member of the major component sterols also in the leaf intracellular lipids, although sitosterol was predominant in these lipids. The surface lipids of immature rape green siliques contained a large proportion of cholesterol.

The sterol compositions of sterol ester, free sterol, acylsterylglycoside and sterylglycoside in hexane extracts of rape leaves are given in Table 2. No marked difference in their sterol compositions was observed among these lipid classes, in which cholesterol was the principal sterol. The hexane extracts of rape leaves consisted mainly of the wax components such as hydrocarbons, wax esters including sterol esters, triacylglycerols, free fatty acids, free aliphatic alcohols and free sterols; their detailed compositions will be reported elsewhere. It is unclear whether sterylglycoside and acylsterylglycoside existed as the wax components or as the contaminants by partial extraction of the intracellular lipids.

Identification of cholesterol and cholestanol by GC-MS and other methods. The peak agreeing with that of authentic cholesterol on gas chromatogram was further characterized by GC-MS. Figure 2 shows a mass spectrum of the peak identified on a gas chromatogram as TMS cholesterol from rape leaf lipids. Characteristic fragment ions of TMS cholesterol appeared on this spectrum, which agreed with that reported in the literature (22). GC-MS data of rape and other plant leaf sterols as free sterols also showed that one of the detected major sterols was cholesterol which gave the typical ions at m/z 386 (M^+),

TABLE 1

Sterol Composition of Rape Leaves, Seeds and Siliques (%)

Sterol	RRT[a]	Leaves		Seeds		Siliques
		Surface	Intracellular	Surface	Intracellular	Surface
Cholesterol	1.00	71.5	14.9	7.2	0.7	34.7
Cholestanol	1.02			1.7	0.2	0.4
Unknown 1	1.05	5.9	7.7	2.1		0.8
Brassicasterol	1.07	4.0	12.0	2.2	6.3	1.4
Campesterol	1.18	8.7	10.1	18.9	18.8	15.7
Stigmasterol	1.24	9.3	19.2	5.7	5.2	25.7
Unknown 2	1.29	tr	3.9			tr
Sitosterol	1.36	0.6	30.4	62.2	67.3	21.3
Unknown 3	1.41	tr	1.8		1.5	
Sterol content (mg/100 g)		34.6	65.7	4.3	11.5	20.4

[a]RRT, relative retention time of TMS derivatives to TMS cholesterol on ULBON HR-52 capillary column (0.24 mm i.d. × 50 m).

tr, trace (<0.2%).

TABLE 2

Sterol Composition of Rape Leaf Lipid Classes Containing Sterols (%)

Major sterol	RRT[a]	Sterol ester	Free sterol	Acylsteryl-glycoside	Steryl-glycoside
Cholesterol	1.00	77.1	61.6	66.6	62.0
Unknown 1	1.05	0.4	5.3	5.7	7.4
Brassicasterol	1.07	2.1	3.0	5.6	7.1
Campesterol	1.18	6.4	10.7	8.9	8.3
Stigmasterol	1.24	13.3	9.9	12.4	13.7
Sitosterol	1.36	0.7	9.5	0.8	1.5

[a]Relative retention time (RRT) is the same as that in Table 1.

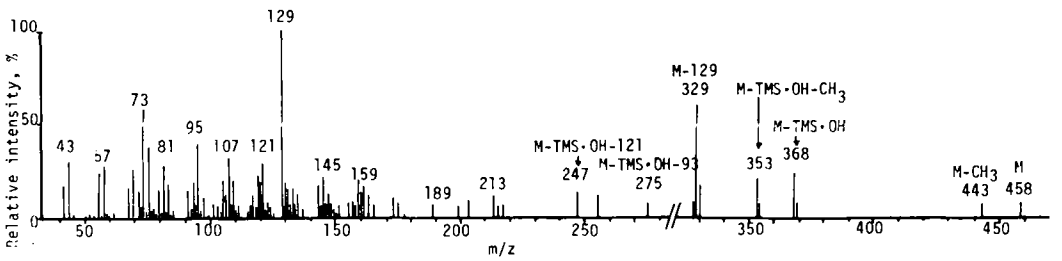

FIG. 2. Mass spectrum of the TMS sterol detected and identified as TMS cholesterol on gas chromatogram of rape leaf surface sterols as their TMS derivatives. A glass column (2 mm × 1 m) packed with 1% OV-1 on Chromosorb W was used at 245 C for GC separation and ionizing voltage was 70 eV.

371 ($M^+ - CH_3$), 368 ($M^+ - H_2O$), 301 ($M^+ - 85$), 275 ($M^+ - 111$), 273 (M^+ − side chain), 213 ($M^+ - 173$), etc., agreeing with those of authentic cholesterol (Fig. 3) (11).

GC-MS analysis of the plant surface sterols further proved that these plant sterol fractions often contained cholestanol as a minor component sterol that was inseparable from cholesterol on a GC-packed column. For example, a GC-MS spectrum of the peak of cholesterol in spinach leaf sterols is shown in Figure 3A. It was found that the cholesterol was contaminated with a minor amount of cholestanol, giving the characteristic ions at m/z 388 (M^+), 373 ($M^+ - CH_3$), 275 (M^+ − side chain), 233 (M^+ − side chain − C_3H_6), 215 ($M^+ - 173$), etc. These findings coincided with those in capillary GC by which cholesterol and cholestanol were separated from each other and differentiated (Figs. 1A and 1D).

A fraction corresponding to cholesterol was separated on reversed-phase TLC from the sterol mixture of rape

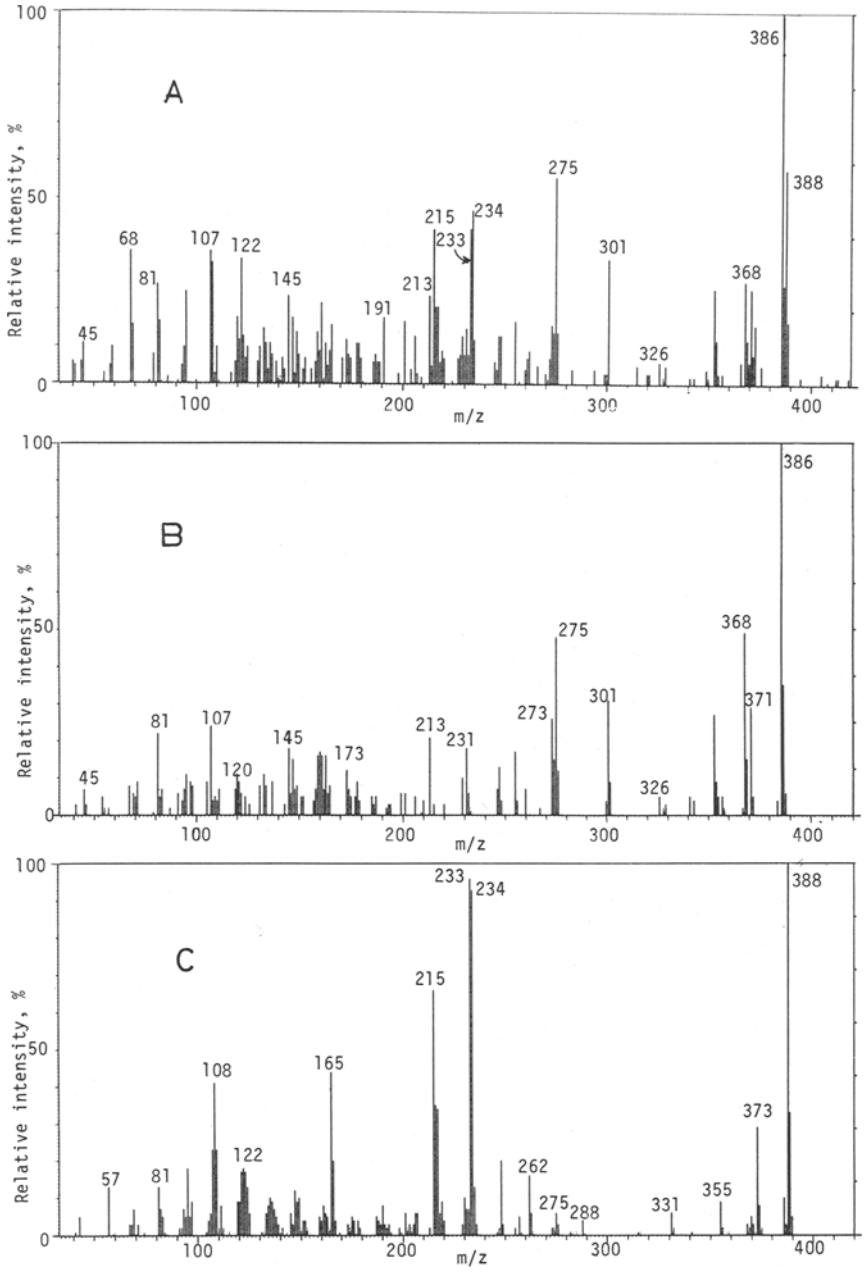

FIG. 3. Mass spectra of free sterols. The spectra were taken at the peak corresponding to that of cholesterol on a GC column (3 mm × 1 m) packed with 2% OV-101 on Chromosorb WHP at 260 C. Ionizing voltage was 20 eV. A, a cholesterol fraction from surface lipids of spinach leaves; B, standard cholesterol; C, standard cholestanol.

leaf surface lipids. The main component of this fraction was characterized as cholesterol by capillary GC, and the ^{1}H NMR spectra showed the presence of Δ^5 double bond (δ 5.34 ppm, br. *s*, 1H, =C<u>H</u>- at C-6) and absence of both Δ^7 and Δ^{22} double bonds, because the olefin protons at C-22 and 23 (δ 5.09, *m*, 2H) and at C-7 (δ 5.15, br. *s*, 1H) (11) were not detectable. In addition, the presence of the C-7 methylene protons could be confirmed by use of downfield shift plots of the proton signals by incremental addition of Eu(fod)$_3$ to the sample. An IR spectrum of the fraction showed the absorptions (KBr disk) at 3400, 2940, 2860, 1660, 1460, 1380 and 1060 cm^{-1}, which were characteristic of sterol.

From these analytical data, the sterol agreeing with authentic cholesterol in its GC retention time was identified as cholesterol.

Distribution of cholesterol in plant species. The sterol compositions of leaf surface lipids in various plant species other than rape are listed in Table 3, in which the sterol compositions of some green immature fruits also are given in comparison with those of green leaves. Cholesterol was found to be one of the major component sterols of leaf surface lipids in the different plant species examined and its proportion reached over 10% in most cases with few exceptions. Even in spinach and chrysanthemum leaves, cholesterol remained as a major sterol, although

TABLE 3

Sterol Composition of Plant Surface Lipids[a] (%)

Plant	Cholesterol	Cholestanol	Brassicasterol	Campesterol	Stigmasterol	Sitosterol	Other sterols[b]
Leaves							
Spinacia oleracea (Spinach)	16.4	3.6		4.4	18.4	35.3	21.9[c]
Brassica oleracea (Cabbage)	4.7	1.1		26.3		67.2	0.7
Rhaphanus sativus (Radish)	28.2		2.3	11.4	5.5	45.0	7.6
Chrysanthemum coronarium	10.1	1.4	2.2	1.8	5.2	50.2	29.1
Allium tuberosum (Leek)	28.6	4.7		4.0	49.0	13.7	
Viburnum awabuki	31.4	6.9		13.6	3.9	34.8	9.4
Fruits							
Vicia faba (Broad bean)	32.9	1.5		26.9	2.7	28.3	7.7
Abelmoschus esculentus (Okra)	17.5	4.5		18.2	16.3	43.5	
Capsicum annuum (Green pepper)	1.1		2.5	38.2	9.8	48.4	

[a]The composition of sterols represents that of the recovered sterols from the unsaponifiable matter after alkali hydrolysis.
[b]Unidentified sterols of Δ^5- and Δ^7-series.
[c]Contains spinasterol and other Δ^7-sterols.

these species were expected to contain considerable amounts of Δ^7-sterols. Similarly, many of the fruit surface lipids seem to contain cholesterol as a common sterol component. However, the cholesterol contents were exceptionally small in cabbage leaves and green pepper fruits. Cholestanol also was detected as a minor component of leaf and fruit surface sterols in many plant species.

DISCUSSION

The present results demonstrate that the majority of the plant species examined contain a considerable proportion of cholesterol in the component sterols of leaf and fruit surface lipids. Therefore, cholesterol seems to be a common and important component sterol also in plants. These facts up to now have been overlooked, presumably because plant sterol compositions have been estimated generally with seed lipids that contain cholesterol as a minor component sterol.

It is probable that cholesterol may be present as a common major sterol also in the fruit surface lipids of various plant species with few exceptions. The detailed data on the sterol compositions of fruit surface lipids will be reported in the near future. A remarkable difference in the cholesterol content was observed even in the same plant genus, as shown between rape and cabbage (Tables 1 and 3).

In Table 1, the proportion of cholesterol was lower in the leaf intracellular lipids than in the surface lipids, in spite of a high proportion of cholesterol in chloroplast sterols as reported by Knights (16). This may suggest the specific localization of cholesterol among cell organelles. It is not known if the chlorophyll content of leaves is responsible for the cholesterol content. Further experiments may be necessary on the changes in cholesterol content in the leaf surface lipids during development of leaves, and also on the mechanism of the localization of cholesterol on the leaf surface.

The biosynthesis of ecdysteroids by insects has been discussed generally on the assumption that cholesterol as the precursor of ecdysone may be metabolized mainly from 24-alkylsterols such as sitosterol, stigmasterol, campesterol, etc., because cholesterol was regarded as the minor component sterol in plant leaves (23). However, the direct synthetic route from cholesterol in leaf lipids may not be negligible in consideration of the presence of a significant amount of cholesterol in leaf sterols as reported here.

Analytical data on the sterol compositions of vegetable foods should be reexamined from the viewpoint of the distribution and localizatoin of cholesterol as a major sterol component in vegetables.

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REFERENCES

1. Fedeli, E., Lanzani, A., Capella, P., and Jacini, G. (1966) *J. Am. Oil Chem. Soc. 43*, 254-256.
2. Itoh, T., Tamura, T., and Matsumoto, T. (1973) *J. Am. Oil Chem. Soc. 50*, 122-125.
3. Kornfeldt, A., and Croon, L.-B. (1981) *Lipids 16*, 306-314.
4. Garg, V.K., and Nes, W.R. (1984) *Phytochemistry 23*, 2919-2923.
5. Garg, V.K., and Nes, W.R. (1984) *Phytochemistry 23*, 2925-2929.
6. Cattel, L., Balliano, G., Caputo, O., and Delprino, L. (1980) *Phytochemistry 19*, 465-466.

7. Akihisa, T., Thakur, S., Rosenstein, F.U., and Matsumoto, T. (1986) *Lipids 21,* 39-47.
8. Fenner, G.P., Patterson, G.W., and Koines, P.M. (1986) *Lipids 21,* 48-51.
9. Heupel, R.C., and Nes, W.D. (1985) *Phytochemistry 24,* 2905-2909.
10. Heupel, R.C., and Nes, W.D. (1985) *Phytochemistry 24,* 2929-2937.
11. Heupel, R.C., Sauvaire, Y., Le, P.H., Parish, E.J., and Nes, W.D. (1986) *Lipids 21,* 69-75.
12. Nes, W.R., Krevitz, K., Joseph, J., Nes, W.D., Harris, B., and Gibbons, G.F. (1977) *Lipids 12,* 511-527.
13. Salt, T.A., and Adler, J.H. (1986) *Lipids 21,* 754-758.
14. Palmer, M.A., and Bowden, B.N. (1977) *Phytochemistry 16,* 459-463.
15. Kajimoto, G., Yoshida, H., and Shibahara, A. (1985) *Nippon Eiyo Shokuryo Gakkaishi 38,* 185-189.
16. Knights, B.A. (1971) *Lipids 6,* 215-218.
17. Brandt, R.D., and Benveniste, P. (1972) *Biochim. Biophys. Acta 282,* 85-91.
18. Kemp, R.J., and Mercer, E.I. (1968) *Biochem. J. 110,* 119-125.
19. Sekula, B.C., and Nes, W.R. (1980) *Phytochemistry 19,* 1509-1512.
20. Heftmann, E. (1984) in *Isopentenoids in Plants: Biochemistry and Function* (Nes, W.D., Fuller, G., and Tsai, L.S., eds.) p. 487, Marcel Dekker, New York, NY.
21. Iida, T., Kikuchi, M., Tamura, T., and Matsumoto, T. (1979) *J. Lipid Res. 20,* 279-284.
22. Natale, N. (1977) *Lipids 12,* 847-856.
23. Svoboda, J.A., and Thompson, M.J. (1987) in *Ecology and Metabolism of Plant Lipids* (Fuller, G., and Nes, W.D., eds.) pp. 176-186, American Chemical Society, Washington, DC.

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Premalignant Alterations in the Glycosphingolipids of Small Intestinal Mucosa of Rats Treated with 1,2-Dimethylhydrazine

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1,2-Dimethylhydrazine is a procarcinogen with selectivity for the colon and proximal small intestine. In weekly subcutaneous (s.c.) doses of 20 mg/kg body weight, this agent produces colonic and proximal small intestinal tumors in a high percentage of rodents with a latency period of approximately six months. To determine whether alterations in the glycosphingolipid content of rat proximal and/or distal small intestinal mucosa existed before the development of dimethylhydrazine-induced cancer, rat were given s.c. injections of this agent (20 mg/kg body weight per wk) or diluent for five wk. Animals were killed at this time, and mucosa was isolated from each small intestinal segment of both groups. Glycosphingolipids then were extracted from these tissues and analyzed by high performance thin layer chromatography and gas liquid chromatography.

The results of these studies demonstrated that (1) the content of neutral and acidic glycosphingolipids was significantly decreased (approximately 20%) in the proximal small intestine of treated rats compared with their control counterparts; (2) no significant difference in the glycosphingolipid content was seen, however, in the distal small intestinal mucosa of control and treated rats; and (3) while significant differences were noted in the majority of fatty acids of G_{M3}, glucosyl- and globotriaosylceramide in the proximal small intestine of control and treated animals, differences in the fatty acids of these glycosphingolipids in the distal segment of these groups were confined to stearic (18:0) acid and/or arachidic (20:0) acid.

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During the past several years, the relationship between glycosphingolipids and the malignant transformation process has received considerable attention (1–3). Recently, utilizing the 1,2-dimethylhydrazine model of colonic adenocarcinoma (4,5) our laboratory has demonstrated alterations in the glycosphingolipid composition and content of colonic epithelial cells of rats administered dimethylhydrazine (20 mg/kg body weight/wk) s.c. for five wk (6). Because this treatment regimen produces colonic tumors in virtually 100% of rodents with a latency period of about six months (4,5), these glycosphingolipid changes induced by this procarcinogen were noted before the development of histologically detectable tumors and, therefore, were termed "premalignant." Their exact relationship to the malignant transformation process, however, remained unclear.

In this regard, the dimethylhydrazine model used in our earlier experiments has been studied extensively (4,5). One aspect of this model that generally has not been appreciated, however, is the predilection for this procarcinogen to produce tumors in the proximal small intestine as well as in the colon (4). At a dosage of 20 mg/kg body weight/wk s.c. for 20–24 wk, a significant percentage of rats develop adenocarcinomas of the duodenum and proximal jejunum but not of the ileum (4). Therefore, the present studies were undertaken to examine and compare the glycosphingolipid content and composition of proximal and distal small intestinal mucosa of rats administered diluent or dimethylhydrazine for five wk. The results obtained from these experiments adds support to our earlier contention that alterations in glycosphingolipids may be an early event in the intestinal malignant transformation process induced by 1,2-dimethylhydrazine (6) and serves as the basis for the present report.

MATERIALS AND METHODS

Materials. Sphingosine standards and 3% OV-1 on Anakron ABS were purchased from Analabs (North Haven, CT). Phytosphingosine was obtained from Calbiochem (La Jolla, CA). Florisil (60–100 mesh) and fluorescamine (Fluram-Roche) were obtained from Fisher Scientific (Fairlawn, NJ). Mixtures of nonhydroxy and hydroxy fatty acid standards and EGSS-X 10% on Gas Chrom P and Power-Sil-Prep were obtained from Alltech/Applied Science Lab. Inc. (State College, PA). The ganglioside standards G_{M1}, G_{D1a}, G_{T1a} and G_{T1b} were purchased from Supelco (Belleforte, PA). G_{M2} isolated from Tay-Sachs brain was a gift from G. Dawson. G_{M3} was isolated and purified by the method of Glickman and Bouhours (7) from rat small intestine. Ceramide was purchased from Analabs. Glucosyl-, lactosyl-, globotriaosyl- and globotetraosylceramide were gifts from S. K. Kundu and were purified from human erythrocytes (8). All other chemicals were purchased from Fisher Chemical Co. (St. Louis, MO) or Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Lipid extraction. Male albino Sherman rats weighing 75 to 100 g were given s.c. injections of diluent (24 rats) or 1,2-dimethylhydrazine (Sigma Chemical Co.) (24 rats) at a dose of 20 mg/kg body weight per week for five wk as described (6). After five wk, control (3 groups of 8 rats) and dimethylhydrazine-treated animals (3 groups of 8 rats) were fasted for 18 hr with water ad libitum before being killed. Weight gain was comparable in all groups at this time. The animals were killed by cervical dislocation, and the proximal one-third and distal one-third of the small intestines of each group of animals were excised. The mucosa of each segment was scraped off with a glass slide and pooled with members of its respective group. Each of the samples then was homogenized in a waring blendor in methanol and extracted with 20 volumes of chloroform/methanol (2:1, v/v), overnight (9). The protein residue was collected by filtration and re-extracted with chloroform/methanol (1:2, v/v). The filtrates were

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Abbreviations: GLC, gas liquid chromatography; HPTLC, high performance thin layer chromatography.

combined, dried in a rotary evaporator and redissolved in chloroform/methanol (2:1, v/v) for further analysis (see below).

Glycosphingolipid and ceramide purification and identification by high performance thin layer chromatography (HPTLC). Total lipid extracts were partitioned with water and the lower phase washed five times with Folch upper phase (chloroform/methanol/water; 3:48:47, v/v/v) to insure complete extraction of gangliosides into the upper phase (9). The upper phase gangliosides then were purified by C18 reverse-phase chromatography (Sep-pak cartridges) as described (10).

The neutral glycolipids and ceramide were purified by chromatography of the acetylated neutral lipids on a Florisil column (11). The gangliosides, ceramide and the deacetylated neutral lipids were analyzed by HPTLC on Silica-gel 60 precoated plates (E. Merck, Darmstadt, West Germany) in the solvent system chloroform/methanol/water/2% $CaCl_2$ (60:35:7:1, v/v/v/v) (12). Glycolipids were visualized by α-naphthol-sulfuric acid spray, while gangliosides were visualized by resorcinol spray (13).

Individual neutral glycolipids and ceramide also were identified by brief exposure to iodine vapor and then quantified after scraping the silica gel area corresponding to their position following migration. The amount of sphingoid bases liberated by methanolysis was determined by fluorimetry following reaction of the free bases with fluorescamine (11). Quantification of the various gangliosides was performed using a Beckman densitometer as described by Mullin et al. (12). All of the bands from each ganglioside species were analyzed in the present experiments. Recoveries were determined using radiolabeled glycosphingolipids as described by Suzuki et al. (14). Recoveries for G_{M3}, ceramide, glucosyl-, lactosyl- globotriaosyl- and globotetraosylceramide were 77 ± 6, 82 ± 7, 81 ± 6, 78 ± 4, 85 ± 6 and 79 ± 5%, respectively (N = 3). The variations in recoveries of these various lipids were not found to be significantly different.

Desialylation of G_{M3}. Desialylation of purified G_{M3} was performed by acid hydrolysis using 0.1M H_2SO_4 at 80 C for 1.5 hr as described (15). N-glycolyl and N-acetylneuraminic acids were separated on HPTLC plates using a solvent system of n-propyl-alcohol/water/ammonium hydroxide (60:28:1.5, v/v/v) (16) and visualized with resorcinol.

Analysis of carbohydrate moieties of G_{M3} and neutral glycolipids. Carbohydrate moieties were identified by gas liquid chromatographic (GLC) analysis of their trimethylsilyl derivatives as described (7,17).

Analysis of fatty acids and long-chain bases of glycosphingolipids and ceramide. Individual glycosphingolipids and ceramide were separated and identified by HPTLC (see above) and after scraping the silica gel area corresponding to their position were hydrolized in 1 ml of dry methanolic HCl (0.75 N) at 80 C for 16 hr, followed by extraction of fatty acid methyl esters by hexane (18). The methanolic phase then was alkalinized with aqueous NaOH and the long chain bases extracted by diethyl ether (19).

Nonhydroxy and hydroxy fatty acids were separated by chromatography on a Florisil column as described (20). In certain experiments, a known amount of C_{21} fatty acid methyl ester was added as an internal standard to quantitate the nonhydroxy and hydroxy fatty acids in the samples (21). Nonhydroxy fatty acid methyl esters were dissolved in a small volume of hexane while hydroxy fatty acid methyl esters were silylated before chromatography (21). Analyses of both nonhydroxy and hydroxy fatty acids were performed on a Hewlett-Packard 5790A gas liquid chromatograph equipped with a flame ionization detector and interfaced with a Hewlett-Packard 3390A integrator as described (21). Peak identification was based on identical retention times with known standards run under the same conditions (21).

Long-chain bases, free from fatty acids, were dissolved in chloroform, washed with 0.1 M NaOH and then repeatedly washed with water. The chloroform phase was dried under N_2 and the residue silylated as described (22). The long-chain bases then were analyzed by GLC on an OV-1 column operated with temperature programming from 245 to 305 C at 3 C per min (21). Identification was based on identical retention times with known standards run under the same conditions (21).

Histological studies. At five wk, 1-cm proximal and distal segments from each control and dimethylhydrazine-treated animal immediately were fixed in 4% paraformaldehyde. Fixed specimens then were embedded in paraffin for light microscopic examination and stained with hematoxylin and eosin as described (23).

Statistical methods. Unless otherwise indicated, all results are expressed as mean values ± S.E. Paired or unpaired Student's t-tests were used for all statistical analysis. $P < 0.05$ was considered significant.

RESULTS

Light microscopic studies. In the present studies, despite extensive sampling of both control and treated small intestinal mucosa, no evidence of severe atypia, carcinoma in situ or microscopic adenocarcinoma was seen in either the proximal or distal-thirds of the small intestine after five wk of administration of diluent or dimethylhydrazine (not shown).

Analysis of glycosphingolipids and ceramide in control and dimethylhydrazine-treated small intestinal mucosa. In agreement with earlier studies by our laboratory (24) and others (7,21,25), as assessed by HPTLC the major neutral glycosphingolipids of the control proximal and distal-thirds of the small intestinal mucosa were glucosylceramide and globotriaosylceramide (Table 1), and the major ganglioside was G_{M3} (Table 2). Smaller amounts of lactosyl- and globotetraosylceramide as well as ceramide, a non-sugar-containing compound, also were present in both of these control segments (Table 1). In the dimethylhydrazine-treated segments, these same neutral and acidic glycosphingolipids predominated, and their relative percentages were similar to their control counterparts.

As shown in Tables 1 and 2, however, the overall content of the neutral glycosphingolipids and gangliosides of the proximal segment of dimethylhydrazine-treated rats, as assessed by HPTLC, each were decreased significantly (approximately 20%) compared with the content of these glycosphingolipids in the proximal segment of control rats. In contrast, the content of these lipids in the distal segment was similar in control and treated animals (Tables 1 and 2).

Analysis of the sugar moieties of these glycosphingolipids by GLC further established their identities and

TABLE 1

Content and Relative Percentages of Ceramide and the Neutral Glycosphingolipids of Proximal and Distal Small Intestinal Mucosa of Control and Dimethylhydrazine-treated Rats Assessed by HPTLC

	Proximal third				Distal third			
	Control		Treated		Control		Treated	
Ceramide/glycosphingolipid	Content[a]	%	Content	%	Content	%	Content	%
Ceramide	3.3 ± 0.8	15.9	2.6 ± 0.7	16.2	10.0 ± 1.0	16.6	10.1 ± 1.1	16.5
Glucosylceramide	6.4 ± 0.9	31.5	4.9 ± 1.6	30.3	19.9 ± 2.3	33.1	19.2 ± 1.8	31.5
Lactosylceramide	1.7 ± 0.4	8.1	1.8 ± 0.8	11.5	6.3 ± 0.5	10.6	6.1 ± 0.3	9.9
Globotriaosylceramide	6.3 ± 1.4	30.7	5.2 ± 0.4	32.6	18.5 ± 1.9	30.7	18.8 ± 1.6	30.8
Globotetraosylceramide	2.2 ± 0.7	10.5	1.5 ± 0.8	9.3	5.3 ± 0.9	8.8	6.7 ± 0.8	11.4
Total	20.2 ± 1.0		16.0 ± 1.2*		60.2 ± 1.6		60.8 ± 1.4	

[a]Content values are expressed as nmol sphingosine/mg protein and represent means ± S.E. of three separate preparations (8 rats/prep.) of each group.

*$P < 0.05$ compared with proximal third control value.

TABLE 2

Content and Relative Percentages of G_{M3} and Other Gangliosides of Proximal and Distal Small Intestinal Mucosa of Control and Dimethylhydrazine-treated Rats Assessed by HPTLC

	Proximal third				Distal third			
	Control		Treated		Control		Treated	
Ganglioside	Content[a]	%	Content	%	Content	%	Content	%
G_{M3}	5.6 ± 0.6	84.4	4.6 ± 0.3	86.2	8.5 ± 0.6	88.6	8.3 ± 0.4	85.7
Others	1.0 ± 0.3	15.3	0.7 ± 0.3	13.7	1.2 ± 0.3	11.0	1.4 ± 0.3	14.5
Total	6.6 ± 0.5		5.3 ± 0.4*		9.6 ± 0.5		9.7 ± 0.4	

[a]Content values are expressed as μg NeuAc/mg protein and represent means ± S.E. of three separate preparations of each group.

*$P < 0.05$ compared with proximal third control value.

showed similar molar ratios of these moieties for each of the glycosphingolipids of control and dimethylhydrazine-treated samples of proximal and distal small intestinal mucosa (not shown).

After acid hydrolysis, the sialic acid moieties of G_{M3} of control and dimethylhydrazine-treated proximal and distal segments were found to contain both N-acetyl and N-glycolylneuraminic acid. The latter form, however, predominated in both segments and was approximately equal in control and treated samples (Table 3).

To detect any other possible differences between the glycosphingolipids of control and treated-intestinal mucosa, the long-chain bases and fatty acids of neutral and acidic glycolipids of both segments were examined and compared. Earlier studies by our laboratory (24) and others (21) have shown that the long-chain base of the various glycolipids was predominantly phytosphingosine (4D-hydrosphinganine) in control rat small intestinal mucosa. As shown in Table 4, the principal base of each neutral glycosphingolipid as well as G_{M3} in control and dimethylhydrazine-treated proximal and distal segments also was found to be phytosphingosine, although lesser quantities of sphingosine also were present in each of these glycosphingolipids. No significant differences, however, were noted in the relative percentages of these bases in the various glycosphingolipids of control and treated intestinal mucosal segments examined (Table 4).

In agreement with earlier studies performed in rat small intestinal mucosa (21), G_{M3}, glucosyl- and globotriaosylceramide in both intestinal segments of control animals contained hydroxy and nonhydroxy fatty acids (Tables 5-7). The fatty acids of G_{M3} and glucosylceramide in these tissues were predominantly hydroxylated (Tables 5 and 6), whereas the fatty acids of globotriaosylceramide predominantly were found to be nonhydroxylated (Table 7) (21). In the present studies, the relative percentages of hydroxy and nonhydroxy fatty acids in these three glycosphingolipids were similar in dimethylhydrazine-treated and control preparations (Tables 5-7).

In contrast to these findings, however, analysis of the individual fatty acids of treated and control G_{M3} revealed significant differences, particularly in the proximal intestinal segments (Table 5). As can be seen in this table, the hydroxy and nonhydroxy levels of palmitic (16:0), stearic (18:0), oleic (18:1), arachidic (20:0) and behenic (22:0) acids of G_{M3} in the treated proximal segment differed significantly from their control counterparts.

TABLE 3

Relative Percentage of N-Acetyl and N-Glycolylneuraminic Acid in G_{M3} of Proximal and Distal Small Intestinal Mucosa of Control and Dimethylhydrazine-treated Rats[a]

	Proximal third		Distal third	
Form of sialic acid	Control	Treated	Control	Treated
N-Acetyl	35.3 ± 2.1	34.1 ± 1.4	34.4 ± 2.5	33.9 ± 1.7
N-Glycolyl	64.6 ± 2.2*	65.8 ± 1.5*	63.7 ± 1.7*	66.0 ± 1.7*

[a]Values represent means ± S.E. of three separate preparations of each group.

*P < 0.05 or less compared with N-acetyl value.

TABLE 4

Relative Percentages of Sphingosine (S) and Phytosphingosine (P) in Glycosphingolipids of Different Segments of Small Intestinal Mucosa of Control and Dimethylhydrazine-treated Rats[a]

	Proximal third				Distal third			
	Control		Treated		Control		Treated	
Glycosphingolipid	S	P	S	P	S	P	S	P
Glucosylceramide	11.0	70.1*	12.0	73.1*	11.0	69.2*	13.0	74.1*
Globotriaosylceramide	10.5	74.0*	10.5	76.0*	10.6	73.2*	11.0	75.4*
G_{M3}	11.6	76.0*	10.4	79.6*	11.1	71.1*	9.5	76.0*

[a]Values represent the mean of three separate preparations of each group by GLC (21,26). Percentages do not add up to 100% because of other bases as yet unidentified. All S.E. were less than 7% and therefore are not shown.

*P < 0.01 or less compared with S-value of same control or treated segment.

TABLE 5

Hydroxy and Nonhydroxy Fatty Acid Composition of G_{M3} from Different Segments of the Small Intestinal Mucosa of Control and Dimethylhydrazine-treated rats[a]

	Proximal third				Distal third			
	Control		Treated		Control		Treated	
Fatty acids	HFA % (71.4)	NFA % (28.9)[b]	HFA % (73.3)	NFA % (26.6)	HFA % (70.4)	NFA % (30.0)	HFA % (72.2)	FA % (27.7)
16:0	8.5	15.7	3.5*	9.6*	11.5	17.6	13.5	16.9
16:1	trace	trace	trace	trace	trace	trace	trace	trace
18:0	26.4	29.4	14.5*	19.1*	28.5	36.1	32.0*	26.4*
18:1	5.4	5.5	8.0*	3.5*	5.5	8.0	6.7	6.7
20:0	10.6	9.2	7.8*	6.3*	8.9	7.8	9.8	8.2
20:4	trace	trace	trace	trace	trace	trace	trace	trace
22:0	3.7	10.2	6.4*	8.5*	9.2	8.6	7.0	10.7
24:0	8.2	8.5	8.5	38.1*	7.2	9.2	8.3	9.4
26:0	15.2	4.6	53.0*	—	8.0	—	8.7	—

HFA, hydroxy fatty acids; NFA, nonhydroxy fatty acids.

[a]Values are means of three separate preparations. S.E. were all less than 6% of mean value. Trace indicates 2.0% or less of the total.

[b]Values in parentheses represent the relative percentages of hydroxy or nonhydroxy fatty acids for G_{M3} extracted from each group. Values reported are the means of three separate preparations and were obtained with the use of methylheneicosanate as internal standard. S.E. were all less than 5% of mean values.

*P < 0.05 or less compared with control values of that segment.

TABLE 6

Hydroxy and Nonhydroxy Fatty Acid Composition of Glucosylceramide from Different Segments of the Small Intestinal Mucosa of Control and Dimethylhydrazine-treated Rats[a]

	Proximal third				Distal third			
	Control		Treated		Control		Treated	
Fatty acids	HFA % (70.3)	NFA % (29.6)[b]	HFA % (72.3)	NFA % (27.5)	HFA % (69.1)	NFA % (30.7)	HFA % (71.4)	NFA % (28.5)
16:0	24.7	24.4	23.2	23.2	22.0	23.7	20.7	24.3
16:1	Trace	Trace	Trace	Trace	2.1	2.4	Trace	Trace
18:0	20.1	20.5	32.0*	31.7*	15.0	17.4	32.6*	35.4*
18:1	16.3	15.7	8.9*	9.2*	18.0	19.1	18.9	20.7
20:0	21.7	22.2	14.3*	14.7*	22.0	22.4	9.2*	10.2*
20:4	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
22:0	6.6	6.9	8.1*	8.9*	5.6	5.6	5.8	4.1
24:0	3.2	Trace	2.3	—	4.5	—	5.5	—
26:0	—	—	—	—	3.7	—	2.1	—

HFA, hydroxy fatty acids; NFA, nonhydroxy fatty acids.

[a]Values are means of three separate preparations. S.E. were all less than 5% of mean value. Trace indicates 2.0% or less of the total.

[b]Values in parentheses represent the relative percentages of hydroxy or nonhydroxy fatty acids for glucosylceramide extracted from each group. Values reported are the means of three separate preparations and were obtained with the use of methylheneicosanate as internal standard. S.E. were all less than 5% of mean values.

*$P < 0.05$ or less compared with control values of that segment.

TABLE 7

Hydroxy and Nonhydroxy Fatty Acid Composition of Globotriaosylceramide from Different Segments of the Small Intestinal Mucosa of Control and Dimethylhydrazine-treated Rats[a]

	Proximal third				Distal third			
	Control		Treated		Control		Treated	
Fatty acids	HFA % (28.6)	NFA % (71.4)	HFA % (29.3)	NFA % (70.6)	HFA % (31.3)	NFA % (68.7)	HFA % (31.0)	NFA % (69.1)
16:0	32.7	34.4	37.7*	37.6*	35.6	37.1	36.0	37.0
16:1	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
18:0	19.4	19.3	22.4*	22.1*	16.9	15.9	25.2*	20.8*
18:1	11.5	11.7	7.7*	8.0*	15.8	16.5	15.4	15.5
20:0	21.0	20.3	17.5*	18.2	14.7	17.5	12.5*	10.3*
20:4	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
22:0	5.2	5.3	6.7*	7.1*	4.1	6.7	4.0	5.9
24:0	Trace	Trace	Trace	—	—	2.5	—	2.3
26:0	Trace	Trace	—	—	—	—	—	—

HFA, hydroxy fatty acids; NFA, nonhydroxy fatty acids.

[a]Values are means of three separate preparations. S.E. were all less than 6% of mean value. Trace indicates 2.0% or less of the total.

[b]Values in parentheses represent the relative percentages of hydroxy or nonhydroxy fatty acids for globotriaosylceramide extracted from each group. Values reported are the means of three separate preparations and were obtained with the use of methylheneicosanate as internal standard. S.E. were all less than 6% of mean values.

*$P < 0.05$ or less compared with control values of that segment.

Moreover, marked increases were noted in the levels of nonhydroxy-lignocenic acid (24:0) and hydroxy-cerotic acid (26:0) of G_{M3} in the treated proximal segment (Table 5). In the distal segment, differences in the hydroxy and nonhydroxy levels of the fatty acids of G_{M3} only were seen with respect to stearic acid (Table 5).

Similarly, as shown in Tables 6 and 7 small but significant differences were noted in many of the hydroxy and nonhydroxy fatty acids of glucosyl- and globotriaosylceramide present in proximal treated and control preparations, whereas in the distal segments only the levels of hydroxy and nonhydroxy stearic and arachidic acids were found to differ in these neutral glycosphingolipids of control and treated animals.

DISCUSSION

In an earlier publication (6), our laboratory demonstrated that a number of alterations could be detected in colonic

epithelial cells of rats administered dimethylhydrazine s.c. (20 mg/kg body weight/wk) for five wk. While changes in the acidic and neutral glycosphingolipids of small intestinal mucosa also were noted in the present experiments using this same treatment regimen, it is of interest to compare and contrast the results obtained in these two studies.

In this regard, dimethylhydrazine treatment significantly reduced the total content of glycosphingolipids in the rat colon and proximal small intestine by approximately 11% and 20%, respectively, whereas it did not affect the glycolipid content of the rat distal small intestine. Furthermore, this agent increased the content and relative percentage of colonic globotriaosylceramide and decreased the content and relative percentage of colonic G_{M3} and globotetraosylceramide, while not significantly affecting these glycosphingolipids in either segment of rat small intestine. In both small and large intestines, dimethylhydrazine did not appear to influence the relative percentages of the long-chain bases of the major glycosphingolipids of these organs or the relative percentage of the sialic acid moieties of G_{M3}. However, dimethylhydrazine did appear to influence the individual hydroxy and nonhydroxy fatty acids of the principal glycosphingolipids of rat colon and small intestine. It should be noted that the changes induced by this agent involved a number of different fatty acids in the glycosphingolipids of rat colon and proximal small intestine, while in the distal small intestine the changes were confined to stearic and/or arachidic acids. These results deserve further comment.

First, it is important to note that in the present studies the dimethylhydrazine-induced decrease in glycosphingolipid content was confined to the proximal small intestine. Furthermore, while alterations in the individual fatty acids of the major ganglioside, G_{M3}, and the major neutral glycolipids, glucosoyl- and globotriaosylceramide, were detected in both small intestinal segments, the compositional changes clearly were more generalized and impressive in the treated proximal segment. These findings are particularly interesting in view of the predilection for the development of tumors in the proximal small intestine of animals treated with this procarcinogen (4). The specific reason(s) that underlie this increased propensity to develop proximal intestinal neoplasia in this cancer model remain unclear (4), although earlier investigations (4) have suggested that this phenomenon may be related to biliary contents, particularly bile salts, acting as promoters of carcinogenesis at this intestinal site. These results, taken together with our earlier findings, which demonstrated that dimethylhydrazine treatment also produced changes in the composition of glycosphingolipids in the rat colon, strongly suggest that these changes may be involved in the malignant transformation process induced by this agent. Despite extensive sampling, no evidence of severe atypia, carcinoma in situ or microscopic adenocarcinomas was seen in treated or control small or large intestinal (6) tissues in these studies; these data would suggest that these glycolipid alterations may represent "premalignant" alterations.

Second, as summarized by Hakomori (1,3) glycolipid changes associated with oncogenic transformation appear to involve three categories: incomplete synthesis, neosynthesis and organizational rearrangement of membrane glycolipods. In our earlier studies in dimethylhydrazine-treated rat colon (6), the decreases in G_{M3} and globotetraosylceramide appeared to be due, at least in part, to decreases in the activities of the enzymes responsible for their biosynthesis. In the present studies, while the mechanism(s) responsible for the 20% decrease in glycosphingolipids in treated proximal small intestine are unclear, given the colonic data described above, it would seem reasonable to suggest that these changes may involve a decrease in biosynthesis as well. Clearly, these findings also could be secondary to an increase in degradation. Further studies will be necessary to clarify this issue.

Finally, it should be noted that if one examines the "premalignant" changes in glycospingolipids induced by five wk of administration of dimethylhydrazine in the rat colon and proximal small intestine, no particular pattern of change is seen in both treated tissues. This would suggest that these changes may be organ-specific. However, it will be of interest to determine in future experiments whether longer periods of administration of dimethylhydrazine (10–15 wk) at times before the development of tumors (24–26 wk) produce additional changes in the glycosphingolipids in these organs. If a consistent pattern of "premalignant" changes can be detected in both tissues at these later time points, such changes may provide additional and potentially important information on the mechanism(s) involved in the malignant transformation process induced by dimethylhydrazine.

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REFERENCES

1. Hakomori, S.I. (1975) *Biochim. Biophys. Acta 417,* 55–89.
2. Siddiqui, B., Whitehead, J.S., and Kim, Y.S. (1978) *J. Biol. Chem. 253,* 2168–2175.
3. Hakomori, S.I. (1981) *Annu. Rev. Biochem. 50,* 733–764.
4. LaMont, J.T., and O'Gorman, T.A. (1978) *Gastroenterology 74,* 1157–1169.
5. Ward, J.M. (1974) *Lab. Invest. 30,* 505–513.
6. Dahiya, R., Dudeja, P.K., and Brasitus, T.A. (1987) *Cancer Res. 47,* 1031–1035.
7. Glickman, R.M., and Bouhours, J.F. (1976) *Biochim. Biophys. Acta 424,* 17–25.
8. Suzuki, A., Kundu, S.K., and Marcus, D.M. (1980) *J. Lipid Res. 21,* 473–477.
9. Folch, J., Lees, M., and Sloane STanley, G.H. (1957) *J. Biol. Chem. 226,* 497–509.
10. Williams, M.A., and McCluer, R.H. (1980) *J. Neurochem. 35,* 266–269.
11. Saito, T., and Hakomori, S.I. (1971) *J. Lipid Res. 12,* 257–259.
12. Mullin, B.R., Poore, C.M.B., and Ruppe, B.H. (1984) *J. Chromatogr. 305,* 512–513.
13. MacMillan, V.H., and Wherrett, J.R. (1969) *J. Neurochem. 16,* 1621–1624.
14. Suzuki, A., Kundu, S.K., and Marcus, D.M. (1980) *J. Lipid Res. 21,* 473–477.
15. Siddiqui, B., and Hakomori, S.F. (1973) *Biochim. Biophys. Acta 330,* 147–155.
16. Leeden, R., Salsman, K., and Cabrera, M. (1968) *Biochemistry 7,* 2287–2295.
17. Reinhold, V.N. (1972) *Methods Enzymol. 25,* 244–248.
18. Esselman, W.J., Laine, R.A., and Sweeley, C.C. (1972) *Methods Enzymol. 28,* 140–156.

19. Sweeley, C.C., and Moscatelli, E.A. (1959) *J. Lipid Res. 1,* 40-47.
20. Bouhours, J.F. (1979) *J. Chromatogr. 169,* 462-465.
21. Bouhours, J.F., and Glickman, R.M. (1977) *Biochim. Biophys. Acta 487,* 51-60.
22. Carter, H.E., and Gaver, R.C. (1967) *J. Lipid Res. 8,* 391-395.
23. Brasitus, T.A., Dudeja, P.K., and Dahiya, R. (1986) *J. Clin. Invest. 77,* 831-840.
24. Dahiya, R., and Brasitus, T.A. (1986) *Lipids 21,* 112-116.
25. Bouhours, J.F., and Glickman, R.M. (1976) *Biochim. Biophys. Acta 441,* 123-133.
26. Bouhours, J.F., and Guignard, H. (1979) *J. Lipid Res. 20,* 897-907.

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Analysis of Cardiac Membrane Phospholipid Peroxidation Kinetics as Malondialdehyde: Nonspecificity of Thiobarbituric Acid-reactivity[1]

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When exposed to xanthine oxidase (superoxide)-dependent, iron-promoted Fenton chemistry, purified cardiac membranes evidenced, by the thiobarbituric acid (TBA) test, a virtually instantaneous peroxidative response with a maximal linear rate of 5.8 nmol malondialdehyde (MDA)-equivalents/mEquivalents lipid ester reacted/min. Yet when the lipids purified from these same membranes and reconstituted into liposomes were peroxidized under identical reaction conditions, the TBA test indicated that a pronounced (~20-min) lag period preceded a maximal peroxidation rate of only 2.1 nmol MDA-equivalents/mEquivalents lipid ester reacted/min. After 120 min of peroxidation, the cardiac membranes yielded some 300 nmol TBA-reactive MDA-equivalents/mEquivalent ester, whereas the isolated membrane lipids evidenced ~40% less TBA-reactivity. To verify that these quantitative and kinetic differences in membrane (phospho)-lipid peroxidation occurred with removal of the lipids from their membrane milieu, the MDA produced during both cardiac membrane peroxidation and the peroxidation of the lipids derived therefrom was isolated as its free anion by ion-pair high-pressure liquid chromatography. As quantified spectrophotometrically, true MDA production during myocardial membrane peroxidation was identical in kinetics and in amount to the production of TBA-reactive substance from the peroxidized isolated membrane lipids. These results demonstrate that significant non-MDA, TBA-reactive species are generated during the peroxidation of cardiac membranes, especially before the maximal rates of bona fide MDA production. As a direct consequence, artifactual levels and kinetics of membrane lipid peroxidation do result.
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Free radical-mediated membrane lipid peroxidation through Fenton-type, metal-promoted oxygen chemistry is increasingly being appreciated as a causative pathogenic factor, especially in myocardial injury (2). Consequently, importance rests with the reliable detection and quantitative assessment of the extent and dynamics of membrane lipid peroxidation. The most extensively employed method for the detection and analysis of peroxidation is the thiobarbituric acid (TBA) test, predicated upon the reactivity of a colorless aldehyde end-product of lipid peroxidation, malondialdehyde (MDA), with TBA to produce a red adduct (3). The convenience, ease and and rapidity of the TBA test have tended, in practice, to mitigate against such considerations as the nonspecificity of TBAs chemical reactivity (4), the low efficiency of fatty-acid hydroperoxide breakdown to MDA (5), and the effects that procedural modifications have upon color development (6). The TBA test has been employed repeatedly in a variety of cardiac oxidative injury models to measure myocardial membrane lipid peroxidation (e.g., 7,8).

This report details quantitative and kinetic investigations of the relationship between TBA-reactivity and bona fide MDA production during the peroxidation of cardiac membranes and their lipids. A defined, physiologically relevant oxidative injury system based upon xanthine oxidase (XOD)-mediated, superoxide ($O_2^{\cdot-}$)-dependent, iron-promoted Fenton chemistry (2) is employed. The results demonstrate that only when isolated cardiac membrane lipids are peroxidized does TBA-reactivity reflect MDA production and, hence, lipid peroxidation. The fact that no qualitative or quantitative reliability could be demonstrated to support the use of the TBA test as an analytical tool with which to monitor the peroxidation of the cardiac membranes themselves would urge that caution be exercised when applying the TBA test to membrane/membraneous organelle systems.

EXPERIMENTAL PROCEDURES

Materials. Allopurinol, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), dithiothreitol, tris(hydroxymethyl)aminomethane (Tris), hydroxylamine hydrochloride, ADP, TBA, and tetramethoxypropane were from Sigma Chemical Co. (St. Louis, MO). XOD (analytical preparation from bovine milk; 1.0 unit/mg) and superoxide dismutase (SOD; analytical preparation from bovine erythrocytes; 5000 units/mg) were purchased from Boehringer-Mannheim (Indianapolis, IN). Desferrioxamine B (Desferal) was a gift from Ciba A.G. (Basle, Switzerland). α-Tocopherol was synthesized by Hoffmann-La Roche. All solvents were of analytical grade (Burdick and Jackson, Muskegon, MI).

Isolation of rat myocardial membranes. The procedure was carried out in a dehumidified cold-room (3 C). Conscious male Sprague-Dawley rats (~275 g; Charles River, Boston, MA) maintained on a normal rodent diet were decapitated. Each heart was rapidly excised and perfused via the aorta with 20.0 ml ice-cold 10 mM HEPES buffer, pH 7.4. The trimmed ventricular muscle masses were pooled, minced, and homogenized (100 mg tissue/ml ice-cold HEPES) for 3 × 5 sec with a Tissumizer (Tekmar, Cincinnati, OH) at maximal setting. The homogenate was filtered through four-ply cheesecloth, and the myocardial membranes were isolated from the filtrate by utilizing KCl extraction to remove contractile protein and differential centrifugation in 10.0 mM Tris-HCl, pH 7.4, containing 2.0 mM dithiothreitol (9). The membranes were used immediately after having been washed twice with 25.0 ml 10 mM HEPES-0.145 M KCl, pH 7.4.

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Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MDA, malondialdehyde; $O_2^{\cdot-}$, superoxide anion radical; SOD, superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1); TBA, thiobarbituric acid; Tris, tris(hydroxymethyl)-aminomethane; XOD, xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2); HPLC, high pressure liquid chromatography.

Lipid extraction and quantification. Lipids were extracted and purified from the myocardial membranes by a modified Bligh-Dyer (10) procedure (11). The final chloroform phase containing the purified lipid represented quantitative recovery of myocardial membrane lipid: >98% of the radioactivity associated with the organic fraction obtained by direct saponification (12) of membrane metabolically labeled in vivo from [^{14}C]acetate, sodium salt, (54.0 mCi/mmol, sp. act. [New England Nuclear, Boston, MA]) was recovered in the saponified fraction from the lipid extracted out of an identical amount of membrane. The myocardial membrane total lipid extract was resolved into its constituent phospholipid and neutral lipid fractions by Sep-Pak silica column chromatography (Waters, Milford, MA) (13). All lipids were stored in chloroform under argon at −20 C (14).

Lipid phosphate was determined microchemically on perchloric acid digests (15). The hydroxamate reaction was used to measure lipid ester (16); L-α-phosphatidylcholine dipalmitate (Avanti Polar Lipids, Birmingham, AL) was used as standard.

Preparation of cardiac liposomes. A known amount of myocardial membrane lipid in chloroform was placed into a rotating glass flask and taken to dryness under nitrogen at room temperature. The lipid was taken up in 10 mM HEPES-0.145 M KCl, pH 7.4, and was resuspended by indirect anaerobic sonication for 15 min at room temperature. The liposomes were used immediately.

Peroxidation reaction system. Cardiac liposomes or membranes (at equal lipid concentrations) were subjected to $O_2^{\cdot-}$-dependent, iron-promoted peroxidation in glass tubes. Ninety minutes before the start of the reaction, a chelate was formed in 10.0 mM HEPES-0.145 M KCl buffer, pH 7.4, between Fe^{3+} (1.0 mM $FeCl_3$, final conc.) and ADP (10.0 mM, final conc.) with continuous stirring at room temperature. The peroxidation reaction, in a final volume of 1.0 ml, contained the following components at their specified final concentrations: 10 mM HEPES-0.145 M KCl, pH 7.4; 1.0 mM hypoxanthine; 0.1 mM Fe^{3+}-1.0 mM ADP chelate; 125 μg lipid (as membrane or liposomal suspension); and 10 mUnits XOD. The reaction was started by adding the XOD, mixing, and incubating at 37 C in a shaking water bath. Membrane/liposome samples also were incubated in parallel to the same final lipid concentration but without free radical generator (i.e., without hypoxanthine, Fe-ADP, and XOD). The peroxidation incubation was ended on ice by lipid extraction, acidification, or SOD addition, depending upon the subsequent analysis (below). Solutions of α-tocopherol were prepared under yellow lighting in ethanol such that the final solvent concentration in the peroxidation reaction was 1,000-fold below that which affected peroxidation. Desferrioxamine and allopurinol were solubilized in 10 mM HEPES-0.145 M KCl, pH 7.4. Dose-response curves to define, quantitatively, the effects of these agents were generated with the programming assistance of RS/1 software (BBN Corp., Cambridge, MA) on an IBM PC-AT (IBM, Boca Raton, FL).

Determination of the production of conjugated dienes and TBA-reactive substance during peroxidation. For conjugated diene analysis, lipids were extracted (11) out of 2.0 ml of peroxidation reaction mixture and the parallel, nonperoxidized membrane or lipid samples. The purified lipids were dissolved in 1.0 ml spectro-grade cyclohexane. Spectra of the lipids were taken against cyclohexane from 190 nm–400 nm in a DU-7 kinetic spectrophotometer (Beckman, Palo Alto, CA). Spectra of the lipids of nonperoxidized liposomes and membranes and of the peroxidation reaction mixture itself were the blank spectra. Raw spectra were corrected for Rayleigh scattering (17), and the nonperoxidized lipid and reagent blank spectra were subtracted from the appropriate experimental, peroxidized lipid spectra to obtain difference spectra. Calculation of net conjugated diene formation was made from the difference spectra using the molar absorptivities given (18).

The TBA test was conducted by the following modification of published methods (5,6,19). The peroxidation incubation was terminated on ice by acidification with 0.15 ml ice-cold 76% TCA-2.3 N HCl per 1.0 ml sample (pH = 2.2), and 0.35 ml of a freshly-prepared mixture of H_2O/7.14 M butylated hydroxytoluene in ethanol/1.51% TBA in 0.2 M Tris, pH 7.0, (1:1:5, v/v/v) was added. After thorough mixing, the samples (final pH = 2.4) were incubated in an 80 C shaking water bath for 30 min. After this time, the tubes were plunged into an ice-water bath, and the TBA test was immediately stopped with 0.5 ml ice-cold 91% TCA followed by 2.0 ml $CHCl_3$. After centrifugation for 30 min at 2000 × g, 4 C, the absorbance of the upper phase at 532 nm was read. Various amounts (9.8–40.0 nmol) of MDA standard, freshly prepared by acidification of 1,1,3,3-tetramethoxypropane with 76% TCA-2.3 N HCl (0.15 ml acid mixture with 1.0 ml suitably diluted tetramethoxypropane), were subjected to the identical TBA test procedure as the basis for constructing a standard curve of TBA-reactivity as MDA-equivalents. Computer-assisted regression analysis of the standard curve was used to quantify the molar amounts of MDA-equivalents in the experimental samples. Net peroxidative production of MDA-equivalents was taken as the difference in TBA-reactivity between peroxidized samples that had been incubated in the complete peroxidation reaction system and non-peroxidized samples that had been incubated in parallel without free radical generator. The TBA reaction per se was not affected by the components of the radical generator, α-tocopherol, desferrioxamine, allopurinol or SOD.

Chromatographic MDA isolation and quantification. When the peroxidation reaction was to be analyzed by high pressure liquid chromatography (HPLC) for MDA content, the peroxidation incubation was stopped by adding SOD to a final concentration of 10^{-7} M and freezing the mixture in dry ice-acetone. MDA was then isolated from the sample by an ion-pair HPLC technique (20) based on the work of Bull and Marnett (21). The HPLC system (Beckman Instruments) was equipped with a μBondapak C_{18} stainless-steel 3.9 mm × 30 cm analytical column preceded by a radially packed C_{18} guard column (Millipore/Waters, Milford, MA). MDA, as the enolate anion, was resolved out of the peroxidation reaction by isocratic elution at 3.0 ml/min with a mobile phase of acetonitrile/50.0 mM myristyltrimethylammonium bromide in 0.9 mM sodium phosphate, pH 6.7 (15:85, v/v). Detection was based on UV absorbance at 267 nm, and quantitation was computerized by peak integration with reference to MDA standard. Net peroxidative production of MDA was taken as the difference in MDA content between peroxidized samples that had been incubated in

the complete peroxidation reaction system and parallel, nonperoxidized samples that had been incubated without free radical generator. MDA for the calibration chromatograms was prepared by hydrolysis of malonaldehyde-bis-diethylacetal (Merck, Darmstadt, Germany) with H_2SO_4, as described (22). Absolute MDA concentration in the solution was checked by UV spectrophotometry at 267 nm with the extinction coefficient of 34,000 M^{-1} cm^{-1} (22).

Assessment of "bound" MDA. To liberate any bound MDA before HPLC analysis, membranes/liposomes were hydrolyzed in base (NaOH) at pH 12.0, 60 C, for 30 min (23). The hydrolysis reaction mixtures then were subjected to rapid ultrafiltration through a YM2 membrane (Amicon, Danvers, MA) to exclude high-molecular-weight (>1000) molecules and the cardiac membranes or liposomes themselves. The clarified filtrates were then neutralized (pH ~7.4) with HCl before HPLC analysis for MDA. Bound MDA was considered the difference in MDA content between parallel samples that had or had not been hydrolyzed. Recovery of standard MDA in this procedure was >96%.

Protein determination. Protein was quantified with a dye-binding microassay (24).

RESULTS

Properties of the isolated myocardial membranes. The biochemical characteristics of the isolated rat-heart membranes are summarized in Table 1. The efficiency of myocardial contractile protein extraction by salt was readily apparent from the dramatically lower protein content of the membranes with respect to starting homogenate. In contrast, the membranes represented over 70% of the heart-muscle homogenate lipid. The high recovery of cardiac phospholipid, localized virtually exclusively in membrane (25), and the three-fold increase in lipid:protein ratio of the membranes over homogenate indicate that the isolated membranes were representative of the myocardial membranes in situ. Additional support for this conclusion rests with the finding that, upon sucrose density-gradient subfractionation of the myocardial membranes (26), ~90% of the total membrane phospholipid was found associated with mitochondria, ~7% with sarcoplasmic reticulum and ~3% with sarcolemma; this distribution is reminiscent of the relative membrane areas of the three organelles in the intact heart muscle cell (27).

Conjugated diene production and TBA-reactivity during myocardial membrane and membrane lipid peroxidation. With exposure of isolated cardiac membranes and the total membrane lipid or phospholipid but not neutral lipid to $O_2^{\cdot-}$-dependent, iron-promoted oxy-radical chemistry, net formation of lipid conjugated dienes was observed (Fig. 1). Because in all cases diene formation was normalized to the actual amount of lipid reacted, the parallelisms in both the pattern and level of lipid conjugated diene production from the intact cardiac membranes and from the purified membrane total lipid and phospholipid would indicate that the phospholipid was the membrane target for oxidative damage. By 120 min of reaction, the levels of diene intermediates decreased when, presumably, the propagation phase of peroxidation ended as conjugated

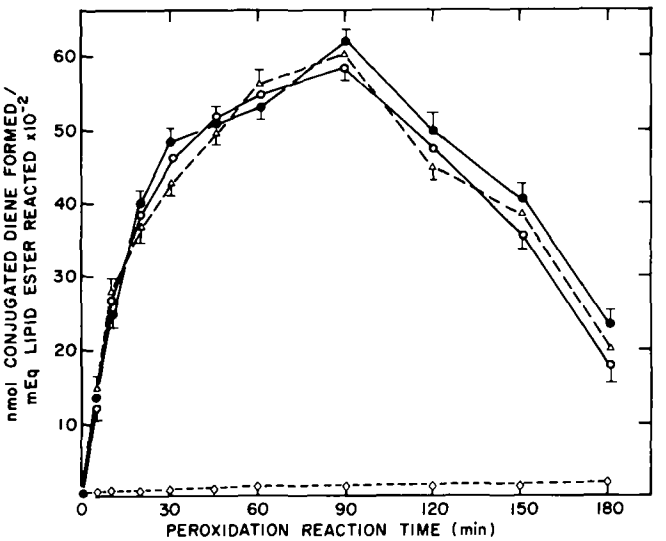

FIG. 1. Conjugated diene production during myocardial membrane and membrane lipid peroxidation. Isolated myocardial membrane (●—●) and the membrane total lipid (○—○), phospholipid (△—△) and neutral lipid (◇—◇) purified therefrom were subjected to peroxidative injury by exposure to XOD ($O_2^{\cdot-}$)-dependent, iron-promoted Fenton chemistry. Samples were taken over 180 min of reaction, and the lipids were extracted and analyzed spectrophotometrically for conjugated diene content. The mean difference in conjugated diene content between the peroxidized samples and non-peroxidized membrane/lipid controls was calculated as the conjugated diene formed during peroxidation. These values, normalized to the amount of lipid reacted, are graphed ± S.D. (n ≥ 6).

TABLE 1

Biochemical Properties of Rat Myocardial Membranes[a]

	Protein		Lipid				
			Ester		Phosphate		
Fraction	(%)	(mg/heart)	(%)	(mEquivalents/heart)	(%)	(μg/heart)	Lipid:Protein
Heart muscle homogenate	100	49.8 ± 2.9	100	6.9 ± 0.3	100	312.0 ± 18.5	0.15 ± 0.01
Myocardial membranes	24.1 ± 0.5	12.0 ± 1.0	72.5 ± 2.6	5.0 ± 0.3[b]	73.2 ± 1.9	228.5 ± 18.4	0.45 ± 0.02

[a]Myocardial membranes were isolated from rat-heart ventricular muscle essentially as described (9). Results are means ± S.D. (n = 6).
[b]90.5 ± 2.6% of the lipid ester is found in membrane phospholipid; the remainder is associated with neutral lipid.

intermediates increasingly converted to products such as lipid peroxides and MDA (5,6).

Exposure of myocardial membranes or liposomes comprised of either the total membrane lipid or the membrane phospholipid to free radical generator resulted in the production of TBA-reactive substance (Fig. 2). As was the case with conjugated diene production (Fig. 1), myocardial membrane neutral lipids evidenced a negligible peroxidative response. However, each of the other myocardial substrates for oxy-radical attack, in contrast to their similar patterns of diene production, showed markedly distinctive kinetics of TBA-reactivity. The intact myocardial membranes evidenced a linear propagation phase through the first 30 min of reaction with an apparent maximal peroxidation rate of 5.8 ± 0.3 nmol MDA-equivalents produced/mEquivalents lipid ester reacted/min (mean ± S.D.; n = 6) and an accumulation of some 300 nmol MDA-equivalents by 180 min. The total myocardial membrane lipid complement, however, displayed a conspicuous lag-period of about 20 min, whereupon an apparent maximal peroxidation rate of 2.1 ± 0.1 nmol MDA-equivalents produced/mEquivalents lipid ester reacted/min led to a net accumulation of about 150 nmol MDA-equivalents by 180 min. Production of TBA-reactive substance from the isolated myocardial membrane phospholipid began virtually instantaneously upon exposure to oxy-radicals, with a linear propagation rate and a net accumulation of TBA-reactive substance that approximated those of the total myocardial membrane lipid.

The lag-period in the production of TBA-reactivity from myocardial membrane total lipid is an effect of the high

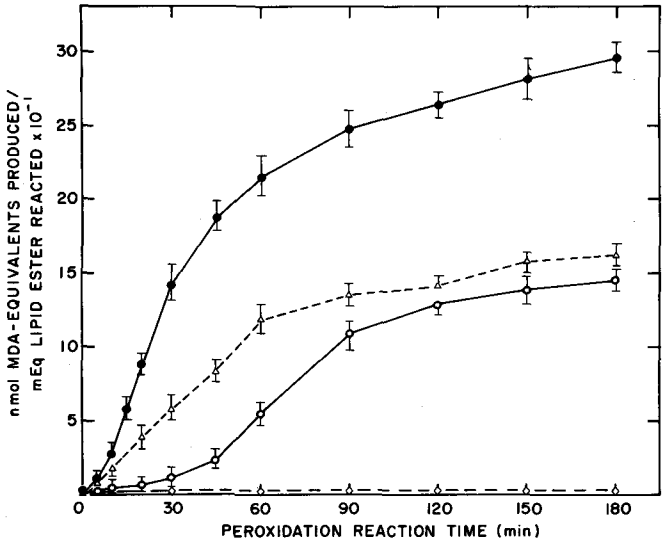

FIG. 2. Generation of TBA-reactive substance during myocardial membrane and membrane lipid peroxidation. Isolated myocardial membranes (●—●) and the membrane total lipid (○—○), phospholipid (△--△) and neutral lipid (◇--◇) purified therefrom were peroxidized by incubation with XOD + hypoxanthine + iron. Membranes/lipids also were incubated in parallel without free radical generator. Samples taken over 180 min of peroxidation reaction were subjected to the TBA test, and the content of TBA-reactive substance in each sample was expressed as MDA-equivalents and normalized to the amount of lipid reacted. The mean difference in MDA-equivalents between peroxidized and non-peroxidized samples was calculated as the MDA-equivalents produced during peroxidation and graphed ± S.D. (n ≥ 6).

myocardial membrane α-tocopherol content (28); the peroxidized isolated membrane phospholipids demonstrated an instantaneous TBA response (Fig. 2). Nonetheless, upon exposure to oxy-radical generator, the membranes, containing both the phospholipids and the α-tocopherol, always generated TBA-reactive substance more rapidly and to a greater extent than did the membrane phospholipids.

HPLC analysis of myocardial membrane peroxidation as MDA. The comparative TBA-reactivity data (Fig. 2) appeared to indicate that profound quantitative and kinetic changes in myocardial membrane phospholipid peroxidation resulted from removal of the lipid out of its membrane milieu. To attempt to verify this conclusion, TBA-reactivity was obviated by isolating MDA throughout the course of myocardial membrane and membrane lipid peroxidation. To avoid the conditions (acid, heat) of the TBA reaction and to ensure complete independence from TBA-reactivity, isolation of, for example, a TBA-MDA adduct was not carried out.

We first defined an effective means of stopping the peroxidation reaction other than by the acidification protocol required for the TBA test, so that precise end-points could be established without jeopardizing subsequent analyses. As shown in Table 2, the XOD ($O_2^{\cdot-}$)-dependent, iron-promoted Fenton chemistry supporting oxidative injury in this system could be blocked at several critical points. Inhibiting XOD with a substrate analog (allopurinol [29]), chelating the iron in the system with desferrioxamine (30), intercepting lipid radicals that would otherwise propagate peroxidation with α-tocopherol (31), and dismutating $O_2^{\cdot-}$ with SOD (32) all were effective means of blocking the generation of TBA-reactive substance from isolated cardiac membrane lipids exposed to XOD + hypoxanthine + iron. The system was most sensitive to SOD: 10.0 nM SOD completely inhibited the generation of TBA-reactive material (as well as the formation of conjugated dienes) from cardiac lipids. Consequently, SOD was added to a final concentration of 100.0 nM to the peroxidation reaction system to dismutate the $O_2^{\cdot-}$ therein and thereby halt the progression of oxidative damage before HPLC analysis for MDA.

To isolate the MDA anion directly out of the peroxidation reaction system without preparatory sample manipulation or clean-up, a variety of amino-phase (33), reverse-phase (34), size-exclusion (35) and ion-pairing (21) HPLC techniques were tested. Ion-pairing chromatography appeared to have the most potential, but the exact conditions of Bull and Marnett (21) offered incomplete resolution of MDA standard from the reactants in the Fenton peroxidation system. With modification of the chromatographic conditions as detailed under Experimental Procedures, MDA could be separated rapidly from the constitutents of the Fenton reaction (Fig. 3) and eluted as a well-resolved peak, 7.0 pmol being the lower-limit of MDA quantitation. Only the material eluting from the column at ~4.9 min evidence TBA-reactivity, had the UV spectrum characteristic of MDA (Fig. 3, inset), and co-eluted with standard MDA. Recovery of known amounts of MDA standard subjected to HPLC isolation was >98%.

With these data, the HPLC technique was adopted to determine directly the MDA produced during oxidative injury to myocardial membranes and their purified lipids

TABLE 2

Inhibition of the Generation of TBA-reactive Substance from Myocardial Membrane Lipid[a]

Agent	Action	Concentration of agent that inhibits TBA-reactivity by: 50%	100%
Allopurinol	XOD inhibition	5.0×10^{-4} M	5.0×10^{-2} M
Desferrioxamine	Iron chelation	—[b]	1.0×10^{-4} M
α-Tocopherol	Chain-breaking antioxidant	8.5×10^{-7} M	1.0×10^{-5} M
SOD	$O_2^{\cdot-}$ dismutation	5.0×10^{-9} M	1.0×10^{-8} M

[a]Liposomes comprised of total myocardial membrane lipid were exposed to XOD + hypoxanthine + iron as radical generator for 60 min either in the absence (control) or presence of varying concentrations of each listed agent. After one hr, the samples were run in the TBA test, and the level of TBA-reactive substance in each was expressed relative to the control samples as inhibition of TBA-reactivity.

[b]Because of the stoichiometric nature of iron chelation by desferrioxamine (30), only complete inhibition was noted at a desferrioxamine concentration equal to the iron concentration in the system.

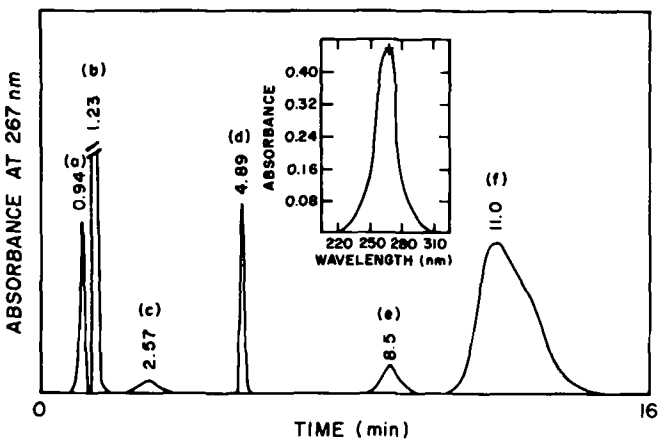

FIG. 3. HPLC isolation of MDA produced during myocardial membrane lipid peroxidation. Myocardial membrane total lipid (125 μg lipid) was reacted with XOD + hypoxanthine + iron in the Fenton peroxidation system described (Experimental Procedures). At one hr of reaction, SOD was added (100 nM, final concentration), and 250 μl of the 1.0 ml sample was subjected to ion-pair chromatography with spectrophotometric detection at 267 nm. Retention times are given above each peak in minutes. The identities of the peaks are (a) hypoxanthine, (b) xanthine, (c) uric acid, (d) MDA, (e) Fe-ADP, (f) ADP. Inset shows the on-line spectrum of the MDA in peak (d) as taken during its elution from the HPLC column; the absorption maximum is indicated by the cross at 267 nm.

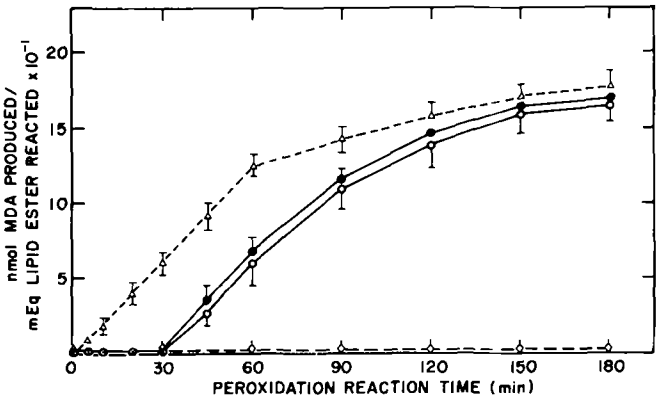

FIG. 4. Production of MDA during myocardial membrane and membrane lipid peroxidation. Isolated myocardial membranes (●—●) and the membrane total lipid (○—○), phospholipid (△—△) and neutral lipid (◇—◇) purified therefrom were peroxidized by exposure to XOD ($O_2^{\cdot-}$)-dependent, iron-promoted Fenton chemistry with XOD + hypoxanthine + iron as oxy-radical generator. Membranes/lipids also were incubated in parallel without free radical generator. Samples were taken over 180 min of peroxidation and were analyzed for their content of MDA (as its enolate anion) by ion-pairing HPLC (Fig. 3). The mean difference in MDA content between peroxidized and non-peroxidized samples was calculated as the MDA produced during peroxidation. MDA production was normalized to the amount of lipid reacted and graphed ± S.D. ($n \geq 6$).

(Fig. 4). Production of MDA during the peroxidation of purified myocardial membrane total lipid, phospholipid or neutral lipid was equivalent in kinetics and magnitude to the accumulation of TBA-reactive substance (expressed as MDA-equivalents; Fig. 2). However, MDA production during peroxidation of isolated myocardial membranes paralleled the MDA production from the purified cardiac membrane lipids (Fig. 4) and did not resemble the levels or pattern of membrane TBA-reactivity (Fig. 2). Because both the TBA-reactivity and MDA production were normalized to the amount of lipid reacted, these results would indicate that non-lipid related TBA-reactive substance generated during myocardial membrane peroxidation was responsible for the apparent attenuation in cardiac membrane lipid peroxidation when the lipids were extracted out of the membranes.

The non-lipid related TBA-reactivity was generated with its own kinetics, as could be demonstrated from the difference curve between TBA-reactivity (as MDA-equivalents) and MDA production upon exposure of myocardial membranes to oxy-radical generator (Fig. 5). It is evident that non-lipid related TBA-reactive substance was generated most rapidly early in the exposure of membranes to the radicals (before ~20 min of reaction) and

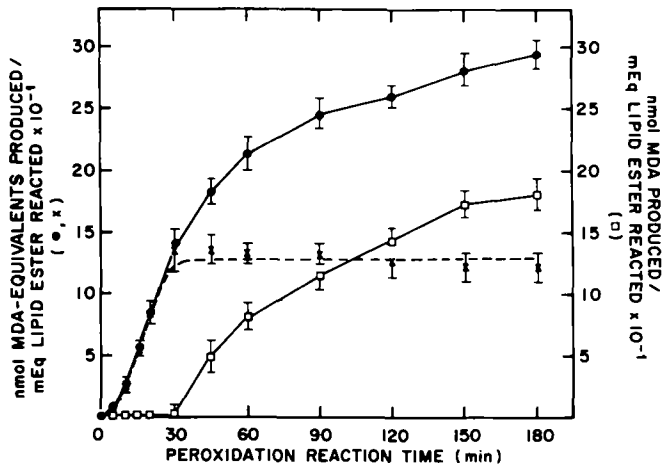

FIG. 5. Production of non-lipid associated TBA-reactive substance during myocardial membrane peroxidation. The generation of TBA-reactive substance (● — ●) and the production of MDA (□—□) during myocardial membrane peroxidation by Fenton-type oxy-radical chemistry are re-plotted from Figs. 2 and 4, respectively. The mean difference (± range) between the amount of TBA-reactive substance and the actual amount of MDA at each sampled time during the 180 min of reaction was calculated and plotted (×——×) as non-lipid related TBA-reactive substance.

ultimately accounted for some 40% of the total TBA-reactivity of the system.

Membrane source of non-lipid related TBA-reactive substance. Several attempts were made to define chemically the source of non-lipid associated TBA-reactivity when myocardial membranes are peroxidized. The acidic conditions and heat of the TBA test will liberate MDA bound to membrane constituents (especially peptide lysine residues) by hydrolyzing Schiff-base linkages (36), whereas the linkages are stable under the mild conditions (no heat) of both the peroxidation reaction (pH = 7.4) and the HPLC run (pH = 6.8). Consequently, additional studies were undertaken in which samples of peroxidized and non-peroxidized membranes were hydrolyzed in base (pH = 12.0) with heating before HPLC isolation of MDA from the filtered, neutralized hydrolysates. This procedure would convert any bound MDA to free MDA that then could be detected as HPLC analyte (23). From comparisons between hydrolyzed membrane samples and non-hydrolyzed samples run in parallel in the peroxidation reaction, some 7% (i.e., 12.2 ± 0.6 nmol MDA/mEq lipid ester reacted; mean ± S.D., n = 6) of the total MDA produced during cardiac membrane peroxidation was in the bound form. This level of bound MDA was too low to account for the 40% difference between membrane TBA-reactivity (as MDA-equivalents) and bona fide MDA generated during membrane peroxidative injury. The bound MDA level remained at ~12 nmol/mEq lipid ester during the course of the peroxidation reaction, indicating that the capacity of the isolated membranes to take up free MDA is limited relative to the MDA yields from peroxidation. This conclusion is substantiated by the finding that cardiac membranes (or their isolated lipids) incubated at 37 C with known amounts of free MDA standard in HEPES-KCl buffer, pH 7.4, yield levels of MDA-equivalents (by the TBA test) or free MDA (by HPLC) that were always >90% of those expected from the standard (data not shown).

Because free acylneuraminic acids are known to react with TBA (37) and cardiac membranes contain acylneuraminic acids (38), it was investigated whether membrane acylneuraminic acids could have contributed to the observed TBA-reactivity of peroxidized rat heart-muscle membranes. To this intent, membranes that had been treated with neuraminidase (acylneuraminyl hydrolase from *Clostridium*; EC 3.2.1.18) (39) or that had undergone controlled acid hydrolysis (40), conditions known to liberate sialic acid from membrane glycoconjugates (41), were peroxidized. The treated cardiac membranes evidenced no statistically significant decrease in the levels and rates of TBA-reactivity development with respect to non-treated membranes (data not shown). Peroxidation of the myocardial membrane protein recovered during the extraction of the membrane lipid did not yield appreciable TBA-reactive substance (data not shown).

DISCUSSION

Growing realization that oxidative damage to membrane lipids is critical in the pathogenesis of a variety of diseases (5,7,8) has placed increased importance upon the application and evaluation of methodology with which membrane lipid peroxidation dynamics are assessed. This study explored in quantitative and kinetic terms the relationships between TBA-reactivity and MDA production during myocardial membrane peroxidation through $O_2^{\cdot-}$ (XOD)-dependent, iron-promoted oxy-radical chemistry of the type believed to occur in myocardial ischemia (2). The data demonstrate that while there is a direct and quantitative relationship between the production of TBA-reactive substance and the generation of free MDA during peroxidation of isolated cardiac membrane (phospho)lipids, no such relationship exists when the myocardial membranes are peroxidized. Generation of non-lipid related, non-MDA TBA-reactive substance with its own kinetics during cardiac membrane peroxidation, if unrecognized, would have led to overestimations of the development and extent of lipid peroxidation and an erroneous peroxidative kinetic profile.

Gutteridge (42) has concluded that the close agreement between the production of TBA-reactive substance and the generation of fluorescent MDA-phospholipid complexes during the peroxidation of isolated ox-brain phospholipid validates the TBA test as an indicator of lipid peroxidation. Although brain membranes were not peroxidized by Gutteridge, and MDA was not isolated, his conclusion is supported by the present data on the reliability of the TBA test in assessing the peroxidation of purified myocardial membrane phospholipids.

Warso and Lands (5,43) have documented extensively that human plasma TBA-reactivity bears no relationship to plasma hydroperoxide level. Specifically, 80% of the TBA-positive material in human plasma was found not to have originated from lipid hydroperoxide conversion to MDA. Quantitatively, these high plasma levels of non-lipid related TBA-reactivity are reminiscent of our finding that ~40% of the TBA-reactive substance produced during the peroxidation of myocardial membrane is not bona fide MDA and does not arise directly from membrane phospholipid peroxidation.

The chemistry and precise source(s) of the non-lipid related TBA-reactive substance generated during cardiac

membrane peroxidation remain to be defined. It is possible that molecular interactions/conformational changes at the membrane level induced during free-radical attack are required to incite formation of TBA-reactive substance beyond that which would be formed from the MDA product of membrane lipid peroxidation (44). Such reasoning has been invoked (45) to explain why the conversion of hydroxyl groups to aldehydes at the termini of cell-surface carbohydrate chains enhances the peroxidation of erythrocyte ghosts by periodate.

The direct quantitative comparison between the production of TBA-reactive substance and the generation of MDA during cardiac membrane and membrane phospholipid peroxidation made herein, along with the work of others in nonmembrane systems (5,42,43), would argue that the TBA test lacks the requisite chemical specificity to be used as a reliable measure, or even as an indicator, of lipid peroxidation, unless purified lipid is the sole oxidative substrate in the system. Independent verification of even the qualitative, comparative significance of a positive TBA test would appear to be necessary to use the TBA reaction as anything other than an empirical indicator of the occurrence of (per)oxidation in biological membrane samples.

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REFERENCES

1. Janero, D.R., and Burghardt, B. (1987) *J. Am. Oil Chem. Soc. 44,* 667-668.
2. Jackson, C.V., Mickelson, J.K., Pope, T.K., Rao, P.S., and Lucchesi, B.R. (1986) *Am. J. Physiol. 251,* H1225-H1231.
3. Nair, V., and Turner, G.A. (1984) *Lipids 19,* 804-805.
4. Gutteridge, J.M.C. (1981) *FEBS Lett. 128,* 343-346.
5. Warso, M.A., and Lands, W.E.M. (1984) *Clin. Physiol. Biochem. 2,* 70-77.
6. Asakawa, T., and Matsushita, S. (1979) *Lipids 14,* 401-406.
7. Kramer, J.H., Mak, I.T., and Weglicki, W.B. (1984) *Circ. Res. 55,* 120-124.
8. Herbaczynska-Cedro, K., and Gordon-Majszak, W. (1986) *Pharmacol. Res. Commun. 18,* 321-332.
9. St. Louis, P.J., and Sulakhe, P.V. (1976) *Int. J. Biochem. 1,* 547-558.
10. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
11. Janero, D.R., and Barrnett, R. (1981) *Anal. Biochem. 111,* 283-290.
12. Brockerhoff, H. (1963) *J. Lipid Res. 4,* 96-99.
13. Juaneda, P., and Rocquelin, G. (1985) *Lipids 20,* 40-41.
14. Janero, D.R., and Barrnett, R. (1981) *J. Chromatogr. 216,* 417-422.
15. Letters, R. (1964) *Biochem. J. 93,* 313-316.
16. Skidmore, W.D., and Entenman, C. (1962) *J. Lipid Res. 3,* 356-363.
17. Watson, B.D., Busto, R., Goldberg, W.J., Santiso, M., Yoshida, S., and Ginsberg, M.D. (1984) *J. Neurochem. 42,* 268-274.
18. Chan, H.W.-S., and Levett, G. (1976) *Lipids 12,* 99-104.
19. Bernheim, F., Bernheim, M.L.C., and Wilbur, K.M. (1948) *J. Biol. Chem. 74,* 257-264.
20. Janero, D.R., and Burghardt, B. (1986) *J. Am. Oil Chem. Soc. 63,* 476-477.
21. Bull, A.W., and Marnett, L.J. (1985) *Anal. Biochem. 149,* 284-290.
22. Esterbauer, H., Lang, J., Zadravec, S., and Slater, T.F. (1984) *Meth. Enzymol. 105,* 319-328.
23. Lee, H.-S., and Csallany, A.S. (1987) *Lipids 22,* 104-107.
24. Pierce, J., and Suelter, C.H. (1977) *Anal. Biochem. 81,* 478-480.
25. Wheeldon, L.W., Schumert, Z., and Turner, D.A. (1965) *J. Lipid Res. 6,* 481-489.
26. Jaqua-Stewart, M.J., Read, W.O., and Steffen, R.P. (1979) *Anal. Biochem. 96,* 293-297.
27. Page, E., McGallister, L.P., and Power, B. (1971) *Proc. Natl. Acad. Sci. USA 68,* 1465-1466.
28. Janero, D.R., and Burghardt, B. (1986) *Fed. Proc. 45,* 1808.
29. Massey, V., Komai, H., Palmer, G., and Elion, G.B. (1970) *J. Biol. Chem. 245,* 2837-2844.
30. Laub, R., Schneider, Y.-J., Octane, J.-N., Touet, A., and Chrichton, R.R. (1985) *Biochem. Pharmac. 34,* 1175-1183.
31. McCay, P.B. (1985) *Ann. Rev. Nutr. 5,* 323-340.
32. McCord, J.M., and Fridovich, I. (1969) *J. Biol. Chem. 244,* 6049-6055.
33. Esterbauer, H., and Slater, T.F. (1981) *IRCS Med. Sci. Libr. Compend. 9,* 749-750.
34. Kakuda, Y., Stanley, D.W., and van de Voort, F.R. (1981) *J. Am. Oil Chem. Soc. 58,* 773-775.
35. Csallany, A.S., Guan, M.D., Manwaring, J.D., and Addis, P.B. (1984) *Anal. Biochem. 142,* 277-283.
36. Gray, J.I. (1978) *J. Am. Oil Chem. Soc. 55,* 539-546.
37. Warren, L. (1959) *J. Biol. Chem. 234,* 1971-1975.
38. Tsuchihashi, H., Sasaki, M., and Nargatomo, T. (1986) *Biochem. Med. Metab. Biol. 36,* 36-44.
39. Schauer, R. (1978) *Meth. Enzymol. 50,* 64-89.
40. Skoza, L., and Mohos, S. (1976) *Biochem. J. 159,* 457-462.
41. Kobata, A. (1979) *Anal. Biochem. 100,* 1-14.
42. Gutteridge, J.M.C. (1977) *Anal. Biochem. 82,* 76-82.
43. Warso, M.A., and Lands, W.E.M. (1985) *J. Clin. Invest. 75,* 667-671.
44. Jain, S.K. (1984) *J. Biol. Chem. 259,* 3391-3394.
45. Beppu, M., and Kikugawa, K. (1987) *Lipids 22,* 312-317.

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Lack of Protein-mediated α-Tocopherol Transfer between Membranes in the Cytoplasm of Acites Hepatomas

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Transfer-stimulating activity for α-tocopherol and the concentration of α-tocopherol and peroxidized lipids in rat ascites hepatoma cells were compared with those from normal and regenerating liver.

The ability of supernatants from ascites hepatomas (AH-13, AH-60C, AH-109A) to enhance the transfer of α-tocopherol was much lower than that from normal livers. The α-tocopherol per mg protein of supernatant from ascites hepatomas was lower than that from normal liver. Regenerating liver showed almost the same values as normal liver in activity to stimulate the transfer of α-tocopherol and α-tocopherol content of the supernatant. By gel filtration, about 60% of α-tocopherol in the supernatant of normal liver was detected in the fractions containing the 30 K protein, which stimulates transfer of α-tocopherol between membranes, whereas no significant amount of α-tocopherol was detected in 30 K protein fractions of AH-60C supernatant. Little stimulating activity for α-tocopherol transfer was detected in AH-60C, AH-109A and AH-13.

All ascites hepatomas tested contained less arachidonic acid and docosahexaenoic acid than normal and regenerating liver. An absorption peak with maximum intensity at 233 nm, which is due to conjugated dienes, was observed in UV-absorption spectra of ascites hepatoma total lipids, indicating that peroxidized lipids accumulate in these cells. With normal and regenerating liver, no significant peak due to conjugated dienes was detected.
Lipids 23, 459-464 (1988).

α-Tocopherol is widely distributed in animal tissues and may function as a lipid antioxidant (1,2). α-Tocopherol, which is orally administered, is absorbed in the intestinal mucosa and then is transported in various lipoprotein fractions in plasma (3-6). The mechanism of intracellular transport of α-tocopherol taken up by cells, however, has not been clarified. We (7) and Murphy and Mavis (8) have demonstrated that rat liver cytoplasm contains a protein that stimulates the transfer of α-tocopherol between membranes. The molecular weight of this protein was estimated to be about 30,000. Behrens and Madere (9) showed that α-tocopherol might be partly bound to a protein with a molecular weight of 32,000 in rat liver cytoplasm in vivo. There may be a pool of α-tocopherol that binds to the transfer protein in the cytoplasm.

Lipid peroxidation in tissues is prevented by biological antioxidants such as α-tocopherol (10-12) and enzymes such as superoxide dismutase (13) and glutathione peroxidase (14-16). There are reports (17-20) showing that activity of these peroxidation-protective enzymes in tumor cells is lower than that in normal tissues. The role of α-tocopherol in protection against peroxidation in these cells cannot be evaluated now because intracellular transport and distribution of α-tocopherol in hepatoma cells have not been elucidated, although some reports (19,21, 22) have compared α-tocopherol content in microsomes of hepatomas with that of rat liver.

Polyunsaturated fatty acids, which are substrates for lipid peroxidation, are lower than those in normal liver in tumor cells such as ascites hepatomas (23), Novikoff hepatomas (21) and Morris hepatomas (21,22).

In this paper, cytosolic activity to enhance the transfer of α-tocopherol between membranes and the cytoplasmic α-tocopherol concentration, as well as the fatty acid composition of total lipids and the amount of peroxidized lipids were examined with rat ascites hepatomas, normal and regenerating liver.

MATERIALS AND METHODS

Materials. Ascites hepatoma cells (AH-13, free cells; AH-60C, island-forming cells; AH-109A, mixture of island and free cells) were donated by H. Sato of the Sasaki Institute (Tokyo, Japan). D-α-[5-Methyl-^{14}C]tocopherol (9.2 Ci/mol), α-tocopherol and tocopherol derivatives were provided by Eisai Co. (Tokyo, Japan). Stock solution of tocopherols was stored in toluene/ethanol (9:1, v/v) at −20 C, and their purity was checked periodically by Silica Gel G thin layer chromatography. Glycerol-tri[9,10(n)-^{3}H]oleate (500 Ci/mol) and glycerol-tri[^{14}C]oleate (52 Ci/mol) were purchased from the Radiochemical Center (Amersham, England). Egg yolk phosphatidylcholine was prepared by chromatography on aluminum oxide and Unisil (Clarkson Chemical Co., Williamsport, PA). Egg yolk [N-methyl-^{3}H]phosphatidylcholine was synthesized by the method of Stoffel et al. (24). Bovine serum albumin (Fraction V) (Sigma Chemical Company, St. Louis, MO) and Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) also were used.

Preparation of homogenate, supernatant and membrane fractions from rat liver and ascites hepatomas. Donryu female rats (100-200 g) were killed by decapitation, and their livers were perfused with cold phosphate-buffered saline (pH 7.5) and then rapidly excised. The liver was minced with scissors, and an homogenate containing 20% (w/v) liver tissue in cold buffer A (0.25 M sucrose, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.4) was prepared with three passes of a Teflon-glass homogenizer.

Ascites hepatoma cells (AH-13, AH-60C, AH-109A) were injected intraperitoneally into Donryu female rats weighing approximately 100 g ($1-2 \times 10^6$ cells per rat). Cells were harvested from the ascites between five and eight days following injection (AH-13, 5-6 days; AH-60C and AH-109A, 7-8 days). All subsequent operations were performed at 4 C. The ascites containing hepatoma cells were centrifuged at 120 × g for five min, and the supernatant was removed; $2-5 \times 10^8$ cells were obtained from the ascites of each rat. Cells were washed in ice-cold phosphate-buffered saline (pH 7.4) several times. The

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Abbreviation: Buffer A, buffer containing 0.25 M sucrose, 1 mM EDTA and 50 mM Tris-HCl (pH 7.4).

homogenate containing 20% (w/v) hepatoma cells in buffer A was prepared with a Teflon-glass homogenizer. The homogenates were centrifuged at 600 × g for 15 min to precipitate nuclei and cell debris. The supernatants were centrifuged at 105,000 × g for 60 min to obtain supernatant and membrane fractions.

Determination of the transfer of α-tocopherol and phosphatidylcholine from liposomes to mitochondria. The procedure was based on the experiment of Bloj and Zilversmit (25). For study of α-tocopherol transfer from liposomes to mitochondria, liposomes composed of 10 μmol egg yolk phosphatidylcholine, 1.0 μmol dicetylphosphate with a trace of α-[^{14}C]tocopherol (0.1 μCi) and [^{3}H]glyceroltrioleate (1.5 μCi) as a "non-exchangeable" marker were prepared as described (7). Lipids were dispersed in 1.0 ml of buffer A with a vortex mixer. The final concentration of liposomes was 10 μmol phospholipid/ml. The lipid suspension was sonicated for 10 min with a microprobe in a Branson Sonifier (model W 185) at 0 C under a stream of nitrogen. The preparation was centrifuged at 15,000 × g for 15 min, and the supernatant was used. Liposomes (0.2 μmol phospholipid) were incubated with mitochondria (0.5 mg protein) in the presence or absence of the supernatants in 2.0 ml of buffer A. Then the mitochondria were precipitated by centrifugation at 15,000 × g for 15 min, and the sample of supernatants (1.0 ml) was counted in a Packard liquid scintillation counter (model 3255). By this procedure, about 90% of the liposomes were recovered in the supernatant. The percentage transfer of α-[^{14}C]tocopherol from liposomes to mitochondria was calculated from the formula:

$$\left(1 - \frac{^{14}\text{C}/^{3}\text{H of liposomes after incubation}}{^{14}\text{C}/^{3}\text{H of liposomes before incubation}}\right) \times 100\%.$$

The ability of the supernatant to stimulate transfer of egg yolk phosphatidylcholine from liposomes to mitochondria was assayed as follows: liposomes composed of 10 μmol egg yolk phosphatidylcholine and 1.0 μmol dicetylphosphate with trace amounts of 0.2 μCi [^{14}C]glyceroltrioleate and 5.0 μCi [^{3}H]egg yolk phosphatidylcholine were incubated with mitochondria (1.5 mg protein) in the presence or absence of the supernatant. After incubation at 37 C for 30 min, the radioactivity recovered in the liposomes was determined. The percentage transfer of [^{3}H]-egg yolk phosphatidylcholine from liposomes to mitochondria was calculated in a similar way to the transfer of α-tocopherol.

Measurement of α-tocopherol content. α-Tocopherol contents of homogenate, supernatant and membrane fractions were estimated by the method of Thompson and Hatina (26). Tocopherol was extracted with hexane/ethanol (5:1, v/v), injected into the column (Zorbax Sil) and eluted with hexane/dioxane/ethanol (97.6:2:0.4, v/v/v). The flow rate was 1.5 ml/min, and the fluorescence in the eluate was measured at excitation 289 nm and emission 325 nm in Shimazu Fluorescence Spectromonitor (RF-530).

Fractionation of 105,000 × g supernatant by Sephadex G-100 column chromatography. A sample of 105,000 × g supernatant was applied to a column containing Sephadex G-100 equilibrated with 100 mM KCl, 50 mM Tris-HCl buffer (pH 7.5), and the fractions were collected. The intensity of the absorption at 280 nm of the fraction was measured in a Hitachi spectrophotometer (model 200-10) in a cell of 1 cm light path. The stimulating activity of α-tocopherol transfer and α-tocopherol content in the fraction also were measured.

Lipid analysis. Total lipids were prepared from homogenates by the method of Bligh and Dyer (27). Phosphatidylcholine and phosphatidylethanolamine were prepared from the total lipids by chromatography on aluminum oxide and Unisil. The fatty acid composition of total lipids, phosphatidylcholine and phosphatidylethanolamine was determined by gas liquid chromatography (Shimadzu Seisaku-sho, GC-OAP). Lipids were dissolved in 2 ml of methanol in water (1:1, v/v) and hydrolyzed by incubation with 0.27 ml of 85% KOH at 80 C for 60 min. Then, the mixture was cooled, acidified with 6 N HCl and extracted three times with diethyl ether. The extracts were combined, washed three times with 2% KCl and evaporated. The residue was methylated with CH_2N_2, and methyl esters were analyzed by gas chromatography on a column (3 mm × 3 m) of 20% EGSS-X on Chromosorb WAW (Gasukuro Kogyo Co.) at 215 C.

Preparation of regenerating liver. Partial hepatectomy was performed according to the method of Higgins and Anderson (28). Donryu female rats (150 g) were anesthetized with diethyl ether, and the abdomen was opened. The median and laberal lobes of the liver were removed. Rats were killed on the fifth day after partial hepatectomy.

Other analytical methods. Phospholipid phosphorus was determined by the method of Gerlach and Deuticke (29). Protein was determined according to the method of Lowry et al. (30).

RESULTS AND DISCUSSION

The transfer of α-tocopherol from liposomes to mitochondria was determined in the presence of the supernatant from normal liver, regenerating liver and ascites hepatomas (Fig. 1A). Transfer-stimulating activity of the supernatant of regenerating liver was almost the same as that of normal liver. The specific activity of AH-13 supernatant (210 pmol/hr/mg protein) was about one-fourth of that of normal liver (900 pmol/hr/mg protein). The supernatant of either AH-60C or AH-109A showed no appreciable transfer-stimulating activity. Low activity in the supernatant of ascites hepatoma cells was not due to the presence of an inhibitor because the activity of normal liver supernatant was not affected by supplement of supernatant from hepatoma cells (data not shown).

As shown in Figure 1B, the supernatant of normal liver also contained phosphatidylcholine-transfer activity as found by Bloj and Zilversmit (25) and Poorthuis et al. (31). The activity could be detected in supernatants of all hepatoma cells tested. It is noteworthy that the transfer-stimulating activity for phosphatidylcholine in the supernatant of AH-60C and AH-109A, which showed no appreciable transfer activity for α-tocopherol, was 60–70% of that of normal rat liver. The activity in the supernatant of AH-13 was a little higher than that of rat liver.

As shown in Table 1, the α-tocopherol content of ascites hepatoma homogenates was the same as homogenates

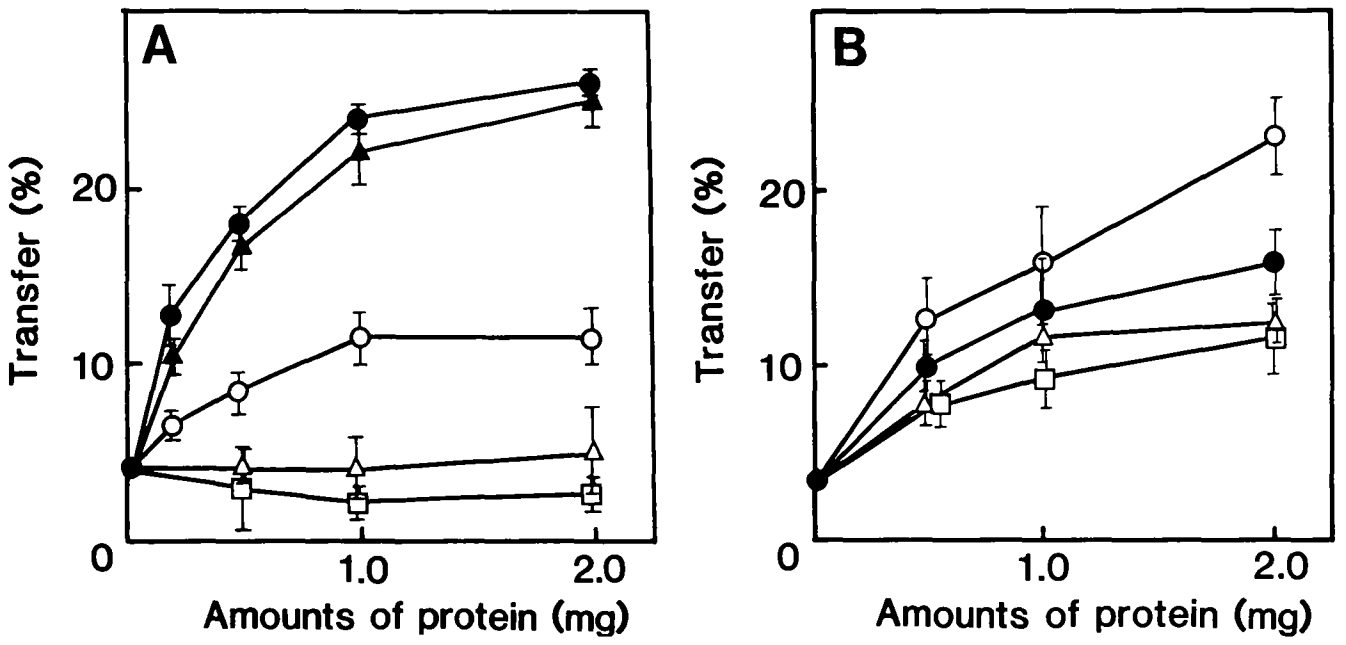

FIG. 1. Transfer-stimulating activity of the supernatant prepared from normal rat liver, regenerating liver or ascites hepatoma cells. (A) Liposomes (0.2 μmol phospholipid) composed of egg yolk phosphatidylcholine, dicetylphosphate (molar ratio, 10:1), a trace of α-[^{14}C]tocopherol and [^{3}H]glyceroltrioleate were incubated with rat liver mitochondria (0.5 mg protein) at 37 C for 15 min in the presence of various amounts of supernatant prepared from normal rat liver (●), regenerating liver (▲) or ascites hepatoma cells (○, AH-13; △, AH-60C; □, AH-109A). The mitochondria were precipitated by centrifugation, and radioactivity of samples of supernatants was measured. Values are expressed as means ± SD of three separate preparations. (B) Liposomes (0.2 μmol phospholipid) comprised of [^{3}H]egg yolk phosphatidylcholine, dicetylphosphate (molar ratio, 10:1) and a trace of [^{14}C]glyceroltrioleate were incubated with rat liver mitochondria (1.5 mg protein) at 37 C for 30 min with the supernatants.

TABLE 1

α-Tocopherol Contents of Homogenate, Supernatant and Membrane Fractions Prepared from Normal Rat Liver, Regenerating Liver or Ascites Hepatoma Cells

	Rat liver		Ascites hepatoma	
Fraction	Normal (nmol/mg protein)	Regenerating (nmol/mg protein)	AH-13 (nmol/mg protein)	AH-60C (nmol/mg protein)
Homogenate	0.20 ± 0.04	0.20 ± 0.06	0.23 ± 0.01	0.19 ± 0.06
Supernatant	0.08 ± 0.01	0.08 ± 0.02	0.02 ± 0.02	0.01 ± 0.01
Membrane	0.40 ± 0.13	0.46 ± 0.07	0.93 ± 0.05	0.45 ± 0.15

To a sample (1 ml) of homogenate, supernatant or membrane fraction prepared from rat liver and ascites hepatomas, 1 ml of ethanol containing 1 nmol tocol (an internal standard) was added. After further addition of 5 ml of hexane, the mixture was mixed vigorously, then centrifuged at 1,800 × g for 10 min. A sample of hexane layer (4 ml) was dried under a stream of nitrogen and dissolved in 50 μl of hexane. The results are means ± SD of the tocopherol content from three separate preparations (one or two rats per preparation).

from normal rat liver (0.2 nmol/mg protein). The α-tocopherol contents in the supernatants of AH-13 or AH-60C were, however, much lower than that of normal liver. The content in the membrane fraction of AH-13 was two times higher than that of normal liver. The value in AH-60C membrane fraction was almost the same as that of normal liver. In regenerating liver, the values in homogenate, supernatant and membrane fractions were almost the same as those of normal liver, respectively.

The supernatants of normal liver and AH-60C also were fractionated by gel filtration. α-Tocopherol transfer activity and α-tocopherol content in each fraction were determined. As shown in Figure 2A, the transfer-stimulating activity in rat liver cytosol appeared in fractions 70–85, whose molecular weight was estimated to be about 30,000, as described (7). About 60% of α-tocopherol in the supernatant also was detected in these fractions. These results are in good agreement with those of Behrens and Madere (9) and suggest that a portion of the cytosolic α-tocopherol exists in the form of a complex with a carrier protein (30 K transfer-stimulating protein). α-Tocopherol also was detected in the fractions just after the void volume. These fractions showed no appreciable activity to stimulate the transfer of α-tocopherol. The amount of

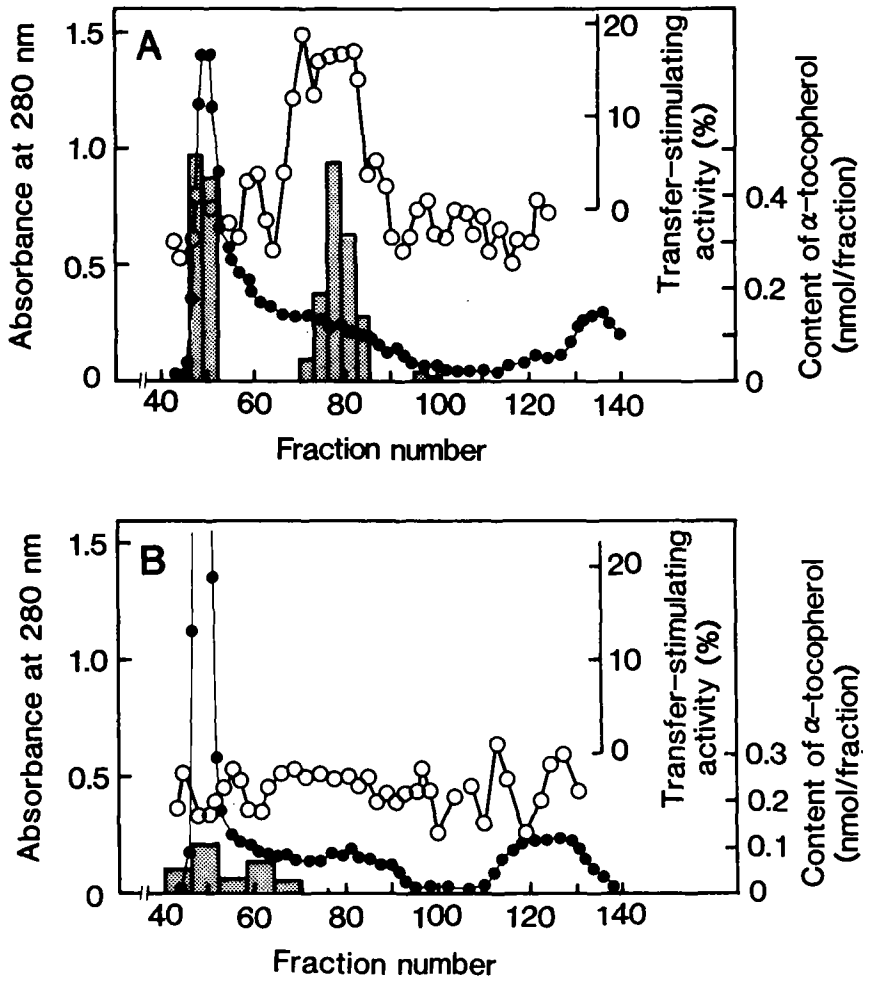

FIG. 2. Distribution of α-tocopherol in the 105,000 × g supernatant. Rat liver cytosol (A) (40 mg protein in 4 ml buffer A) or AH-60C cytosol (B) (20 mg protein in 4 ml buffer A) was applied to a column of Sephadex G-100 (2.6 × 70 cm) equilibrated with 100 mM KCl, 50 mM Tris/HCl buffer (pH 7.5) and eluted with the same buffer at 4 C. The flow rate was about 30 ml/hr, and 4 ml fractions were collected. Elution of protein was determined by measuring the absorbance at 280 nm (●). The transfer-stimulating activity of each fraction (○) was determined as described in Materials and Methods. Transfer-stimulating activity is expressed as the percentage transfer in the presence of each fraction (1 ml) minus the percentage of spontaneous transfer. The α-tocopherol content of each fraction of the supernatant was measured as follows: fractions were combined (12 ml), and 12 ml of ethanol containing tocol (1 nmol) was added. Then, 60 ml of hexane was added and was mixed vigorously. The mixture was allowed to stand at room temperature for five min. A sample of hexane layer (50 ml) was dried with a rotary evaporator, and the residue was dissolved in 50 μl of hexane. A sample of hexane solution (10 μl) was injected into the column. Values are expressed as dotted bars.

labeled α-tocopherol recovered from these fractions was not decreased in the presence of large amount of unlabeled α-tocopherol (7). Behrens and Madere (9) also reported that gel filtration of the rat liver cytosol revealed the presence of a protein with a molecular weight of more than 1,500,000, which binds α-tocopherol with less affinity and specificity. Sklan and Halevy (32) showed that tocopherol was associated with a lipid-protein aggregate with a molecular weight of about 2×10^6 in chick liver cytosol.

With AH-60C supernatant, the amount of α-tocopherol recovered from the 30 K protein fraction by gel filtraton was much smaller than that recovered with rat liver supernatant (Fig. 2B). A significant amount of α-tocopherol was recovered from the fractions whose molecular weight was more than 30,000. None of the fractions, however, showed transfer-stimulating activity. It was concluded that there was no appreciable pool of α-tocopherol bound to the transfer-stimulating protein (30 K protein) in the supernatant of AH-60C.

Decreases in both transfer-stimulating activity and the amount of cytosolic α-tocopherol may not be characteristic of dividing cells because regenerating liver cytosol showed virtually the same transfer-stimulating activity and α-tocopherol content as observed in normal liver cytosol.

Assuming that the 30 K protein plays an important role in distributing α-tocopherol to proper intracellular sites, this suggests that the α-tocopherol apparently associated

TABLE 2

Fatty Acid Composition of Total Lipids of Normal Rat Liver, Regenerating Liver and Ascites Hepatoma Cells

Fatty acid	Normal (wt%)	Regenerating (wt%)	AH-13 (wt%)	AH-60C (wt%)	AH-109A (wt%)
16:0	17.2 ± 1.7	16.8 ± 0.7	18.8 ± 2.1	18.7 ± 2.0	18.1 ± 0.8
18:0	22.1 ± 1.8	25.0 ± 2.5	19.0 ± 2.0	16.9 ± 2.0	19.6 ± 2.0
18:1	12.1 ± 2.9	9.3 ± 0.7	25.6 ± 2.3	31.6 ± 1.1	19.3 ± 2.3
18:2	15.2 ± 0.9	14.6 ± 1.4	24.2 ± 1.2	17.2 ± 0.5	22.2 ± 1.1
20:4	17.7 ± 4.0	16.8 ± 1.2	5.0 ± 1.9	7.0 ± 1.8	8.7 ± 1.6
22:6	7.9 ± 0.5	9.7 ± 1.5	N.D.	2.3 ± 1.3	3.2 ± 0.4
Double-bond index[a]	161	164	94	108	118

[a]The double-bond index was calculated from the sum of the values obtained when the percentage of the unsaturated fatty acid was multiplied by the number of double bonds in the fatty acid (41).

Total lipids (0.2 μmol phospholipid) were hydrolyzed and esterified as described in Materials and Methods. The results are means ± SD of percentage of each fatty acid derived from three separate preparations (one or two rats per preparation).

N.D., not detected.

with membrane fractions of hepatoma cells may not be located on organellar membranes but remain on the plasma membranes or lysosomal membranes. The exact localization of α-tocopherol in tumor cells, therefore, should be examined, although the fractionation of hepatomas as described by various investigators (33–39) is rather difficult now.

The fatty acid composition of total lipids from ascites hepatomas (AH-13, AH-60C, AH-109A) was quite different from that of normal rat liver (Table 2). In ascites hepatomas, the relative amount of polyunsaturated fatty acids, such as arachidonic acid and docosahexaenoic acid, was much smaller than that of normal rat liver. On the other hand, the relative amount of oleic acid in ascites hepatomas was larger than that of normal liver. The lower degree of unsaturation of fatty acids in ascites hepatoma total lipids was indicated clearly by the marked change in the double-bond index. These results are in good agreement with those of Utsumi et al. (23). The composition of regenerating rat liver was almost the same as that of normal rat liver.

Table 3 shows fatty acid composition of phosphatidylcholine and phosphatidylethanolamine prepared from normal rat liver or AH-60C. The fatty acid composition of phosphatidylcholine from AH-60C was quite different from that of rat liver. The arachidonic acid and docosahexaenoic acid content in AH-60C was much lower than that of normal liver. In contrast, the oleic acid content in AH-60C phosphatidylcholine was higher than that of normal liver. AH-60C phosphatidylcholine showed a lower double-bond index as compared with normal liver phosphatidylcholine. However, phosphatidylethanolamine of AH-60C contained almost the same amount of arachidonic acid as that of normal rat liver. The decrease of polyunsaturated fatty acids also was observed in phosphatidylcholine of Morris hepatoma 5123c (40) and with phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol of Yoshida hepatoma AH130 (41). The distribution profile of these polyunsaturated fatty acids in phospholipids may be different among hepatomas.

TABLE 3

Fatty Acid Composition of Phosphatidylcholine and Phosphatidylethanolamine of Rat Liver or AH-60C

	Rat liver		AH-60C	
Fatty acid	PC (wt%)	PE (wt%)	PC (wt%)	PE (wt%)
14:0	4.1	3.1	4.5	3.4
16:0	14.9	18.0	24.3	6.8
18:0	28.7	23.1	13.5	18.1
18:1	6.1	5.4	27.4	19.2
18:2	11.5	9.5	14.9	7.2
20:4	24.5	21.8	9.2	20.3
22:6	7.7	12.4	N.D.	4.6
Double-bond index	173	186	94	142

PC, phosphatidylcholine; PE, phosphatidylethanolamine; N.D., not detected.

Phosphatidylcholine or phosphatidylethanolamine (0.2 μmol phospholipid) prepared by chromatography on aluminum oxide and Unisil was hydrolyzed and esterified as described in Materials and Methods. Values are expressed as percentage of each fatty acid.

Conjugated dienes could be detected in total lipids by measuring the ultraviolet absorption spectrum of the total lipids fraction (Fig. 3). An absorption peak with maximum intensity at 233 nm, which is due to conjugated dienes (42), was observed with total lipids of ascites hepatomas (AH-13, AH-60C, AH-109A). No appreciable peak due to conjugated dienes was detected with total lipids of either normal or regenerating liver of rats. Thus, ascites hepatoma cells appear to contain a larger amount of conjugated diene-containing peroxidized lipids than either normal or regenerating liver.

These results raise the possibility that polyunsaturated fatty acid residues in ascites hepatomas may be more susceptible to peroxidation in vivo than those from normal rat liver. The decrease in the polyunsaturated fatty

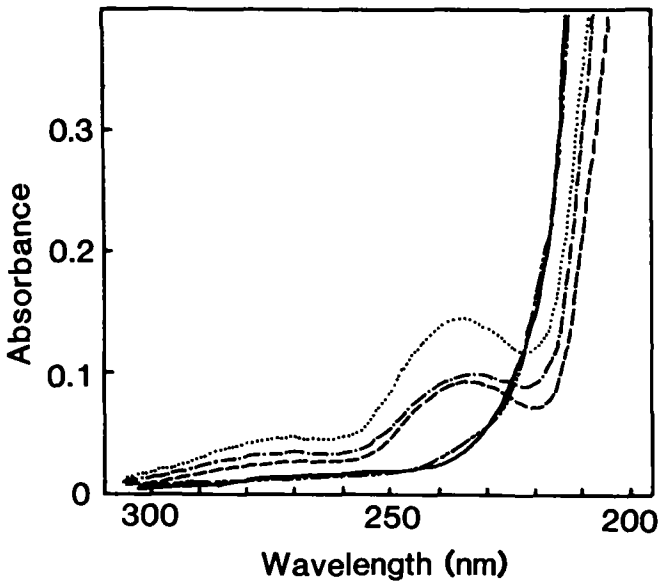

FIG. 3. Ultraviolet absorption spectra of total lipids of rat liver and ascites hepatomas. Total lipids, which were prepared as described in Materials and Methods, were dissolved in ethanol (50 μM phospholipid), and then the spectrum was recorded. ——, Normal rat liver; —··—, regenerating liver; — — —, AH-13; ····, AH-60C; —·—·—, AH-109A.

acid content of hepatoma cells may be due partially to their higher susceptibility to peroxidation of lipids in the cells. The decreases in the transfer-stimulating activity and in the level of α-tocopherol in ascites hepatoma cytosol may be involved in increase of lipid peroxidation in these cells.

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REFERENCES

1. Tappel, A.L. (1972) *Ann. N.Y. Acad. Sci. 203,* 12-18.
2. McCay, P.B., Pfeifer, P.M., and Stipe, W.H. (1972) *Ann. N.Y. Acad. Sci. 203,* 62-73.
3. Rubinstein, H.M., Dietz, A.A., and Srinavasan, R. (1969) *Clin. Chim. Acta 23,* 1-6.
4. Gontzea, I., and Nicolau, N. (1972) *Nutr. Rep. Int. 5,* 225-231.
5. Horwitt, M.K., Harvey, C.C., Dahm, C.H. Jr., and Searcy, M.T. (1972) *Ann. N.Y. Acad. Sci. 203,* 223-236.
6. Kayden, H.J. (1978) in *Tocopherol, Oxygen and Biomembranes* (de Duve, C., and Hayaishi, O., eds.) pp. 131-142, Elsevier Publishing, North-Holland.
7. Mowri, H., Nakagawa, Y., Inoue, K., and Nojima, S. (1981) *Eur. J. Biochem. 117,* 537-542.
8. Murphy, D.J., and Mavis, R.D. (1981) *J. Biol. Chem. 256,* 10464-10468.
9. Behrens, W.A., and Madere, R. (1982) *Nutr. Rep. Int. 25,* 107-112.
10. Tappel, A.L., and Zalkin, H. (1960) *Nature London 185,* 35.
11. McCay, P.B., Poyer, J.L., Pfeifer, P.M., May, H.E., and Gillian, J.M. (1971) *Lipids 6,* 297-306.
12. Witting, L.A. (1980) in *Free Radicals in Biology* (Pryor, W.A., ed.) Vol. 4, pp. 295-319, Academic Press, New York.
13. Fridovich, I. (1976) in *Free Radicals in Biology* (Pryor, W.A., ed.) Vol. 1, pp. 239-277, Academic Press, New York.
14. Coen, G., and Hochstein, P. (1963) *Biochemistry 2,* 1420-1428.
15. Little, C., and D'Brien, P.J. (1968) *Biochem. Biophys. Res. Commun. 31,* 145-150.
16. Chiu, D.T.Y., Stults, F.H., and Tappel, A. (1976) *Biochim. Biophys. Acta 445,* 558-566.
17. Bozzi, A., Mavelli, I., Finazzi, A., Strom, R., Wolf, A.M., Mondovi, B., and Rotilio, G. (1976) *Mol. Cell. Biochem. 10,* 11-76.
18. Peskin, A.V., Koen, Y.M., and Zbarsky, I.B. (1977) *FEBS Lett. 78,* 41-45.
19. Bartoli, G.M., Bartoli, S., Galeotti, T., and Bertoli, E. (1980) *Biochim. Biophys. Acta 620,* 205-211.
20. Tisdale, M.J., and Mahmoud, M.B. (1983) *Br. J. Cancer 47,* 809-812.
21. Cheeseman, K.H., Collins, M., Proudfoot, K., Slater, T.F., Burton, G.W., Webb, A.C., and Ingold, K.U. (1986) *Biochem. J. 235,* 507-514.
22. Borrello, S., Minotti, G., Polombin, G., Grattagliano, A., and Galeotti, T. (1985) *Arch. Biochem. Biophys. 238,* 588-595.
23. Utsumi, K., Goto, N., Kanemasa, Y., Oshioka, T., and Oda, T. (1971) *Physiol. Chem. Phys. 3,* 467-480.
24. Stoffel, W., LeKim, D., and Tschung, T.S. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352,* 1058-1064.
25. Bloj, R., and Zilversmit, D.B. (1977) *J. Biol. Chem. 252,* 1613-1619.
26. Thompson, J.N., and Hatina, G. (1979) *J. Liquid Chromatogr. 2,* 327-344.
27. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
28. Higgins, G.M., and Anderson, R.M. (1931) *Arch. Pathol. 12,* 186-202.
29. Gerlach, E., and Deuticke, B. (1963) *Biochem. Z. 337,* 477-479.
30. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
31. Poorthuis, B.J.H.M., Van der Krift, T.P., Teerlink, T., Akeroyd, R., Hostetler, K.Y., and Wirtz, K.W.A. (1980) *Biochim. Biophys. Acta 600,* 376-386.
32. Sklan, D., and Halevy, O. (1982) *Nutr. Rep. Int. 25,* 499-505.
33. Quintart, J., Bartholeyns, J., and Baudhuin, P. (1979) *Biochem. J. 184,* 133-141.
34. Dyatlovitskaya, E.V., Timofeeva, N.G., and Bergelson, L.D. (1978) *Eur. J. Biochem. 82,* 463-471.
35. Koumanov, K., Boyanov, A., Neicheva, T., Markovska, T., Momchilova, A., Gavazova, E., and Chelibonova-Lorer, H. (1982) *Biochim. Biophys. Acta 713,* 23-28.
36. Schlager, S.I., and Ohanian, S.H. (1979) *Cancer Res. 39,* 1369-1376.
37. Saura, P.L., Trouet, A., and Tulkens, P. (1978) *Biochim. Biophys. Acta 543,* 430-449.
38. Stocco, D.M., and Huston, J.C. (1980) *Cancer Res. 40,* 1486-1492.
39. Schlager, S.I., and Ohanian, S.H. (1980) *J. Immunol. 125,* 508-517.
40. Waite, M., Parce, B., Morton, R., Cunningham, C., and Morris, H.P. (1977) *Cancer Res. 37,* 2092-2098.
41. Ruggieri, S., and Fallani, A. (1979) *Lipids 14,* 781-788.
42. Buege, J.A., and Aust, S.D. (1978) in *Methods in Enzymology* (Fleischer, S., and Packer, L., eds.) Vol. 52, pp. 302-310, Academic Press, New York.

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Effect of 7-Methylated Bile Acids and Bile Alcohols on Cholesterol Metabolism in Hamsters

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The effect of 7-methyl substituted bile acid and bile alcohol analogues on cholesterol metabolism was studied in the hamster. Animals were fed chow plus 0.1% cholesterol supplemented with 0.1% of one of the following steroids: chenodeoxycholic acid, 7-methyl-chenodeoxycholic acid, 7β-methyl-24-nor-5β-cholestane-3α,7α,25-triol, cholic acid, 7-methyl-cholic acid, or 7β-methyl-24-nor-5β-cholestane-3α,7α,12α,25-tetrol. Cholesterol absorption was determined from fecal analysis after feeding of radiolabeled cholesterol and β-sitosterol.

Of the six compounds studied, chenodeoxycholic acid and 7-methyl-chenodeoxycholic acid decreased intestinal cholesterol absorption (17% and 31% decrease, respectively). Only 7-methyl-chenodeoxycholic acid decreased serum cholesterol concentration (29% decrease), but there were no analogous changes of liver and biliary cholesterol concentration and cholesterol saturation of bile. Total fecal neutral sterol excretion was increased in the groups fed chenodeoxycholic acid and 7-methyl-chenodeoxycholic acid. In addition, the production of coprostanol was increased in both groups. These data suggest that 7-methyl-chenodeoxycholic acid resembles chenodeoxycholic acid in its effect on cholesterol metabolism and may be a potential candidate for further studies of its gallstone-dissolving properties.
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Chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) are known to be effective agents for the medical dissolution of gallstones in a selected group of patients (1,2) and in some experimental animals (3). We postulated that introduction of a methyl group at the 7-position of naturally occurring dihydroxy bile acids prevents intestinal bacterial 7-dehydroxylation and, therefore, the production of the potentially hepatotoxic metabolite, lithocholic acid (LCA). Therefore, we synthesized 7-methyl substituted bile acid analogues (4,5) and studied their metabolism in experimental animals (6–9). These studies revealed that 7-methylated bile acids were absorbed and participated efficiently in the enterohepatic circulation of bile acids; they appeared to be resistant to bacterial 7-dehydroxylation.

Recently, Une et al. developed a simple method for the synthesis of 7-methyl bile acids as well as certain C_{26} bile alcohol analogues (Une, M., Yamanaga, K., Mosbach, E.H., Kuroki, S., and Hoshita, T., unpublished data). With this improved technique, it was possible to obtain sufficient quantities of these 7-methyl bile acids and bile alcohols to investigate their chemical and metabolic properties. This study describes the effect of 7-methylated bile acids and bile alcohols on cholesterol metabolism, and particularly on cholesterol absorption, in the hamster.

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Abbreviations: CDCA, chenodeoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; GLC, gas liquid chromatography.

MATERIALS AND METHODS

Unlabeled compounds. Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO). β-Sitosterol (94% pure) was provided by Eli Lilly and Co. (Indianapolis, IN). Cholic acid (CA) (Inolex, Park Forest South, IL) and chenodeoxycholic acid (CDCA) (Weddel Pharmaceuticals, London, U.K.) were greater than 99% pure as determined by gas liquid chromatography (GLC) of the methyl ester-trimethylsilyl ether derivatives. 7-Methyl bile acid and bile alcohol analogues, namely 3α,7α-dihydroxy-7β-methyl-5β-cholanoic acid (7-Me-CDCA), 3α,7α,12α-trihydroxy-7β-methyl-5β-cholanoic acid (7-Me-CA), 7β-methyl-24-nor-5β-cholestane-3α,7α,25-triol (7-Me-NCT), and 7β-methyl-24-nor-5β-cholestane-3α,7α,12α,25-tetrol (7-Me-NCTe), were synthesized as described (4,5, Une, M., Yamanaga, K., Mosbach, E.H., Kuroki, S., and Hoshita, T., unpublished data).

Labeled compounds. [1α,2α(n)-^{3}H]Cholesterol (sp. act. 84.6 mCi/mg) and [4-^{14}C]β-sitosterol (sp. act. 135 μCi/mg) were purchased from NEN Research Products (Boston, MA). The radioactive purity of each compound was better than 97% and 98%, respectively, as determined by radio thin layer chromatography.

Animals and diets. Male golden Syrian hamsters (40 animals) weighing an average of 94 g were purchased from Charles River Breeding Laboratories (Wilmington, MA). All the animals were weighed and placed into individual cages. They all were quarantined for 10 days and fed Purina rodent chow ad libitum. They were then fed a diet of Purina rodent chow (Teklad) containing 0.03% cholesterol and supplemented with 0.1% crystalline cholesterol (group 1). This diet was further supplemented with 0.1% of one of the following (groups 2–7): (2) CDCA; (3) 7-Me-CDCA; (4) 7-Me-NCT; (5) CA; (6) 7-Me-CA; (7) 7-Me-NCTe. The animals were maintained on the different diets and water ad libitum for 12 days. There was no significant weight change in the animals during the experiment. The average food intake of the animals was 7.7 g/day, and there were no significant differences among the seven groups.

The labeled sterols (^{3}H-cholesterol and ^{14}C-β-sitosterol) were diluted with unlabeled carriers to obtain specific activities of 1.1×10^7 cpm/mg and 2.5×10^6 cpm/mg, respectively. The mixture of radioactive sterols was dissolved in 6 ml of acetone and added with stirring to a 30 g portion of each powdered diet (groups 1–7, 5 g/animal). After complete removal of acetone by evaporation, this radiolabeled diet was fed to each animal on day 13 of the experiment. Feces were collected quantitatively on days 14, 15 and 16. The animals then were fed the experimental, steroid-containing diets for an additional eight days after isotopic labeling (days 14–21). At the end of the experiment (day 21), the animals were killed under anesthesia (ketamine hydrochloride and ether). Samples of blood and liver were obtained for the determination of cholesterol concentrations; gall bladder bile was used for the determination of biliary lipids.

Analytical procedures. Determination of cholesterol content of serum, liver and bile was carried out as described (10). Biliary bile acids were quantitated by GLC (8). Bile phospholipids were determined colorimetrically by a procedure described elsewhere (11). Cholesterol saturation of bile was calculated using a computer program (12) based on the tables of Carey (13). The method used for the extraction of the fecal neutral steroids was principally that of Miettinen et al. (14), as modified by Cohen et al. (15). Three-day fecal samples for each animal were pooled. The neutral sterols were extracted with hexane after alkaline hydrolysis, and an aliquot was used for radioisotope measurements. ^{3}H and ^{14}C were determined by liquid scintillation counting (Beckman LS-8000 liquid scintillation system, Beckman Instruments, Fullerton, CA) with the use of external standardization for quench correction. Cholesterol absorption was calculated according to method IV of Quintao et al. (16).

Quantitation of neutral steroids in feces was carried out by GLC of the trimethylsilyl ether derivatives. 5α-Cholestane was added to each sample as an internal standard. The methods and conditions of the GLC analyses were those described (15). All analyses were carried out with a Hewlett-Packard 5830A gas chromatograph.

The results in the tables are expressed as mean ± SE. The significance of differences among various groups studied was calculated by Student's t-test (17).

RESULTS

These studies were carried out to examine the effect of 7-methyl bile acid and bile alcohol analogues on the intestinal absorption of cholesterol and on the activity of those intestinal bacteria responsible for the hydrogenation of cholesterol to coprostanol.

Total recoveries of ^{14}C-β-sitosterol in feces during the three-day collection period are shown in Table 1. The excretion of β-sitosterol was similar in all groups (84.7–88.3%). Calculation of cholesterol absorption was made by method IV of Quintao et al. (16). The results are shown in Table 1. In groups 2 and 3, fed CDCA and 7-Me-CDCA, respectively, cholesterol absorption was reduced in comparison with the control group (group 1, [17% and 31% decrease, respectively]).

Table 2 shows the concentration of serum, liver and biliary cholesterol. The administration of 7-Me-CDCA caused a significant decrease (29%) in serum cholesterol concentration, while 7-Me-CA produced a significant increase (31%), in comparison with the controls. In the groups fed CA or 7-Me-CA, liver cholesterol was increased about two-fold. In contrast, biliary cholesterol levels were similar in all groups.

Cholesterol saturation indices (CSI) of each group are listed in Table 3. Only CA changed the CSI compared with that of control group 1; the decrease produced by

TABLE 1

Recovery of Labeled β-Sitosterol into the Feces and Effect of Various Bile Acids on Intestinal Cholesterol Absorption

Group number	Diet[a]	No. of animals	Fecal dry wt. (g/day)	Recovery of β-sitosterol (%)	Cholesterol absorption (%)
1	Chow + 0.1% CH	5	1.35 ± 0.06	86.4 ± 2.5	71.0 ± 2.6
2	Chow + CDCA	6	1.48 ± 0.04	85.6 ± 2.4	59.2 ± 2.5[b]
3	Chow + 7-Me-CDCA	6	1.38 ± 0.05	88.3 ± 1.4	48.8 ± 8.0[c]
4	Chow + 7-Me-NCT	6	1.39 ± 0.07	86.4 ± 1.1	57.3 ± 7.2
5	Chow + CA	6	1.38 ± 0.08	84.7 ± 1.6	72.8 ± 4.9
6	Chow + 7-Me-CA	6	1.55 ± 0.07[c]	87.7 ± 1.6	58.6 ± 7.7
7	Chow + 7-Me-NCTe	5	1.45 ± 0.08	85.7 ± 3.7	69.0 ± 7.4

[a]The hamsters were fed various bile acids and bile alcohols (0.1%) in a chow diet containing 0.1% cholesterol (CH).

[b]Differ from control, $p < 0.01$.

[c]Differ from control, $p < 0.05$.

TABLE 2

Effect of Bile Acids and Bile Alcohols on Tissue Cholesterol Concentration

Group number	Diet[a]	No. of animals	Serum (mg/dl)	Liver (mg/g)	Bile (mg/ml)
1	Chow + 0.1% CH	5	118 ± 9	11.5 ± 1.7	1.39 ± 0.34
2	Chow + CDCA	6	144 ± 16	14.7 ± 2.2	1.74 ± 0.27
3	Chow + 7-Me-CDCA	6	84 ± 10[b]	10.4 ± 3.4	1.70 ± 0.32
4	Chow + 7-Me-NCT	6	139 ± 18	9.2 ± 2.3	1.38 ± 0.17
5	Chow + CA	6	131 ± 22	22.2 ± 3.3[b]	1.71 ± 0.40[c]
6	Chow + 7-Me-CA	6	154 ± 14[b]	17.8 ± 2.3[b]	1.76 ± 0.28
7	Chow + 7-Me-NCTe	5	106 ± 13	17.7 ± 3.3	1.44 ± 0.20

[a]The hamsters were fed various bile acids and alcohols (0.1%) in a chow diet containing 0.1% cholesterol (CH).

[b]Differ from control, $p < 0.05$.

[c]Five animals.

7-Me-CDCA was not significant. The other compounds had no significant effect on the CSI.

Biliary bile acid composition was determined at sacrifice. In the groups fed CDCA (group 2) and CA (group 5), these bile acids were major components of the bile, 54.6% and 40.9%, respectively. The 7-methylated bile acid analogues, 7-Me-CDCA and 7-Me-CA, composed 19.3% and 13.6%, respectively. The administered bile alcohols, 7-Me-NCT (group 4) and 7-Me-NCTe (group 7), were detected as minor components in bile and comprised 2.2% and 7.8%, respectively.

Daily sterol outputs in feces are shown in Table 4. These measurements were obtained from GLC analyses of the neutral sterol fraction of the feces. CDCA and 7-Me-CDCA increased daily total neutral sterol output, while compounds with the cholic acid-type nucleus (trihydroxy substituents) showed no effect. There were no significant changes in cholesterol output. However, the amount of coprostanol was 1.6- and 2.1-fold higher in the hamsters fed CDCA or 7-Me-CDCA (groups 2 and 3) than in the controls (group 1). We compared the coprostanol/cholesterol ratios to obtain information about the effect of the added steroids (groups 2–7) on intestinal bacterial function. Of the compounds studied, CDCA and its analogues (7-Me-CDCA and 7-Me-NCT), seemed to enhance the formation of coprostanol. The other compounds had no effect.

TABLE 3

Saturation Index of Hamster Bile: Effect of Bile Acids and Bile Alcohols

Group number	Diet[a]	No. of animals	Cholesterol saturation
1	Chow + 0.1% CH	5	0.76 ± 0.11
2	Chow + CDCA	6	1.03 ± 0.08
3	Chow + 7-Me-CDCA	4	0.45 ± 0.22
4	Chow + 7-Me-NCT	6	0.59 ± 0.21
5	Chow + CA	5	0.44 ± 0.08[b]
6	Chow + 7-Me-CA	5	0.72 ± 0.07
7	Chow + 7-Me-NCTe	5	0.46 ± 0.11

[a]The hamsters were fed various bile acids and alcohols (0.1%) in a chow diet containing 0.1% cholesterol (CH).

[b]Differ from control, $p < 0.05$.

DISCUSSION

This study deals with the effect of naturally occurring bile acids, 7-methyl bile acids and C_{26} 7-methyl bile alcohol analogues on cholesterol absorption and other aspects of cholesterol metabolism in hamsters. Several methods for measuring intestinal cholesterol absorption are known (16,18). In this study, we adopted method IV of Quintao et al. (16). Although longer fecal-collection periods are required in the case of human subjects, a three-day fecal collection was considered adequate to measure cholesterol absorption in the hamsters. About 85% of the administered labeled β-sitosterol was recovered during this three-day period (Table 1), probably because of the short intestinal transit time of the hamster. Earlier studies had demonstrated that a 12-day adjustment period was adequate to obtain a steady-state in hamsters (24). Thus, this procedure allows us to obtain a measurement of cholesterol absorption.

Cholesterol absorption studies during bile acid administration revealed that in the rat only cholic acid and its conjugates produced significant increases in cholesterol absorption (19), while monohydroxy or dihydroxy bile acids had no effect (20,21). In this study, administration of CA did not enhance the absorption of cholesterol. It is not clear whether this result is due to the relatively low concentration of dietary cholesterol employed or whether it is characteristic of the hamster model. However, there is controversy whether CDCA alters cholesterol absorption (22). Several studies showed that CDCA had no effect on cholesterol absorption in rat and man (20,23), while other studies suggested an inhibitory effect in man (22). In this report, CDCA and 7-Me-CDCA inhibited the absorption of cholesterol to a significant level in comparison with the controls. Thus, at least in the hamster, dihydroxy bile acids seem to inhibit the intestinal absorption of cholesterol compared with the trihydroxy analogues.

7-Me-CDCA was the only compound in this study that decreased serum cholesterol to a significant extent; this compound did not alter liver and biliary cholesterol compared with controls (Table 2). Moreover, the cholesterol

TABLE 4

Effect of Bile Acid and Bile Alcohols on Fecal Sterol Output

Group number	Diet[a]	No. of animals	Total neutral sterol output (mg/day)	Cholesterol output (mg/g dry feces)	Coprostanol output (mg/g dry feces)	Coprostanol/cholesterol (%)
1	Chow + 0.1% CH	5	3.49 ± 0.45	1.09 ± 0.11	1.50 ± 0.21	1.36 ± 0.05
2	Chow + CDCA	6	5.34 ± 0.38[b]	1.29 ± 0.04	2.42 ± 0.14[b]	1.78 ± 0.13[c]
3	Chow + 7-Me-CDCA	6	6.53 ± 0.99[c]	1.62 ± 0.23	3.08 ± 0.41[b]	1.94 ± 0.19[c]
4	Chow + 7-Me-NCT	6	4.64 ± 0.52	1.18 ± 0.09	2.22 ± 0.36	1.83 ± 0.16[c]
5	Chow + CA	6	3.74 ± 0.54	1.21 ± 0.14	1.52 ± 0.25	1.23 ± 0.12
6	Chow + 7-Me-CA	6	4.71 ± 0.68	1.10 ± 0.06	1.93 ± 0.32	1.72 ± 0.24
7	Chow + 7-Me-NCTe	5	3.46 ± 0.53	0.97 ± 0.04	1.41 ± 0.31	1.43 ± 0.24

[a]The hamsters were fed various bile acids and alcohols (0.1%) in a chow diet containing 0.1% cholesterol (CH).

[b]Differ from control, $p < 0.01$.

[c]Differ from control, $p < 0.05$.

saturation index of bile in this group (group 3) was not affected (Table 3). Similar results were reported earlier in hamsters fed diets containing cholesterol plus UDCA (24). Because 7-Me-CDCA is substituted both in the 7α- and 7β-position, it is possible that it may share certain characteristics of both CDCA and UDCA. It is known, however, that CDCA and 7-Me-CDCA are considerably more hydrophobic than UDCA (4).

Kuroki et al. reported that 7-Me-CA increased serum and liver cholesterol and inhibited HMG-CoA reductase activity in the hamster (25). Although we confirmed that the concentrations of serum and liver cholesterol were increased by the administration of 7-Me-CA, there seemed to be no change in intestinal cholesterol absorption. Thus, it was not possible to demonstrate directly that the inhibition of HMG-CoA reductase by 7-Me-CA was related to the increased hepatic cholesterol concentration caused by increased cholesterol absorption.

Bile alcohol analogues with a methyl group at the 7-position had little effect on cholesterol absorption. We have not examined the metabolism of these compounds in detail, and therefore it is unclear whether these compounds are efficiently absorbed from the intestine, extracted by the liver and excreted into the bile. Kibe et al. reported that in the rat following the intra-abdominal injection of 24-nor-5β-cholestane-3α,7α,12α,25-tetrol, the compound was excreted into the bile as a glucuronide (26). According to Hoshita et al., the intestinal absorption of bile alcohols is not as efficient as that of the bile acids (27). The absorption of the free bile alcohol and bile alcohol 3-glucuronide were 60% and 30%, respectively, during a 24-hr period (27). Therefore, it seems likely that the bile alcohol analogues examined in this study exerted little effect on cholesterol metabolism because they did not participate effectively in the enterohepatic circulation.

It is well-known that bile acid feeding affects intestinal bacterial function (28). In this respect, we showed that 7-Me bile acid analogues diminished the bacterial 7-dehydroxylation of naturally occurring bile acids (25,29). In this present study, we examined the effect of 7-Me compounds on the conversion of cholesterol to coprostanol. Earlier studies had shown that the formation of coprostanol from cholesterol is affected by a number of dietary factors, such as protein, cerebroside, fiber and bile acids (30–33). In this study, 7-Me-CDCA as well as CDCA increased total neutral sterol and coprostanol output; as a result, there was an increase of the coprostanol/cholesterol ratio (Table 4). However, under the conditions employed, it was not possible to state whether these bile acids have a direct effect on the bacterial enzyme(s) involved. Because cholesterol absorption was decreased in these groups (groups 2 and 3), and presumably there was an increased intestinal sterol concentration, it might have been expected that the coprostanol/cholesterol ratio should have decreased (34).

In summary, in this study the most significant effects of the 7-Me-bile acid analogues were observed with 7-Me-CDCA. Administration of the latter produced a decrease in intestinal cholesterol absorption and serum cholesterol concentration and did not affect the biliary cholesterol saturation. In addition, this analogue of CDCA increased fecal neutral sterol output and the coprostanol/cholesterol ratio. Because 7-Me-CDCA prevents the formation of cholesterol gallstones and accelerates their dissolution in the prairie dog model, further studies with this compound seem indicated.

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REFERENCES

1. Fromm, H., Roat, J.W., Gonzalez, V., Sarva, R.P., and Farivar, S. (1983) *Gastroenterology 85,* 1257–1264.
2. Tint, G.S., Salen, G., Colallilo, D., Graber, D., Verga, D., Speck, J., and Shefer, S. (1982) *Ann. Intern. Med. 97,* 351–356.
3. Pearlman, B.J., Bonorris, G.G., Phillips, M.J., Chung, A., Vimadalal, S., Marks, J.W., and Schoenfield, L.J. (1979) *Gastroenterology 77,* 634–641.
4. Une, M., Cohen, B.I., and Mosbach, E.H. (1984) *J. Lipid Res. 25,* 407–410.
5. Kuroki, S., Une, M., and Mosbach, E.H. (1985) *J. Lipid Res. 26,* 1205–1211.
6. Une, M., Singhal, A.K., McSherry, C.K., May-Donath, P., and Mosbach, E.H. (1985) *Biochim. Biophys. Acta 833,* 196–202.
7. Une, M., Mosbach, E.H., Cohen, B.I., May-Donath, P., and McSherry, C.K. (1985) *Lipids 20,* 222–226.
8. Kuroki, S., Mosbach, E.H., Cohen, B.I., and McSherry, C.K. (1987) *Gastroenterology 92,* 876–884.
9. Kuroki, S., Mosbach, E.H., Stenger, R.J., Cohen, B.I., and McSherry, C.K. (1987) *Hepatology 7,* 229–234.
10. Cohen, B.I., Raicht, R.F., and Mosbach, E.H. (1977) *J. Lipid Res. 18,* 223–231.
11. Griswold, B.L., Humoller, F.L., and McIntyre, A.R. (1951) *Anal. Chem. 23,* 192–194.
12. Kuroki, S., Cohen, B.I., Carey, M.C., and Mosbach, E.H. (1986) *J. Lipid Res. 27,* 442–446.
13. Carey, M.C. (1978) *J. Lipid Res. 19,* 945–955.
14. Miettinen, T.A., Ahrens, E.H. Jr., and Grundy, S.M. (1965) *J. Lipid Res. 6,* 411–424.
15. Cohen, B.I., Raicht, R.F., Salen, G., and Mosbach, E.H. (1975) *Anal. Biochem. 64,* 567–577.
16. Quintao, E., Grundy, S.M., and Ahrens, E.H. Jr. (1971) *J. Lipid Res. 12,* 221–232.
17. Snedecor, G.W., and Cochran, W.G. (1946) in *Statistical Methods,* 4th edn., pp. 56–81 and 182–184, Iowa State University Press, Ames, IA.
18. Borgström, B. (1969) *J. Lipid Res. 10,* 331–337.
19. Vahouny, G.V., Gregorian, H.M., and Treadwell, C.R. (1959) *Proc. Soc. Exp. Biol. Med. 101,* 536–540.
20. Raicht, R.F., Cohen, B.I., and Mosbach, E.H. (1974) *Gastroenterology 67,* 1155–1161.
21. Reynier, M.O., Montet, J.C., Gerolami, A., Marteau, C., Crotte, C., Montet, A.M., and Mathieu, S. (1981) *J. Lipid Res. 22,* 467–473.
22. Ponz de Leon, M., Carulli, N., Loria, P., Iori, R., and Zironi, F. (1979) *Gastroenterology 77,* 223–230.
23. LaRusso, N.F., and Thistle, J.L. (1983) *Gastroenterology 84,* 265–271.
24. Singhal, A.K., Finver-Sadowsky, J., McSherry, C.K., and Mosbach, E.H. (1983) *Biochim. Biophys. Acta 752,* 214–222.
25. Kuroki, S., Mosbach, E.H., Cohen, B.I., Stenger, R.J., and McSherry, C.K. (1987) *J. Lipid Res. 28,* 856–863.
26. Kibe, A., Fukura, M., Kihira, K., Kuramoto, T., and Hoshita, T. (1981) *J. Biochem. 89,* 369–377.
27. Hoshita, T., Harada, N., Morita, I., and Kihira, K. (1981) *J. Biochem. 90,* 1363–1369.
28. Floch, M.H., Binder, H.J., Filburn, B., and Gershengoren, W. (1972) *Am. J. Clin. Nutr. 25,* 1418–1426.
29. Hylemon, P.B., Moody, D.P., Cohen, B.I., Une, M., and Mosbach, E.H. (1984) *Steroids 44,* 329–336.
30. Tanaka, C., Watanuki, M., and Nazaki, Y. (1983) *J. Nutr. Sci. Vitaminol. 29,* 447–454.
31. Hansen, I.B., Snog-Kjaer, A., Prange, I., Holmer, G., Sondergaard, E., and Dam, H. (1965) *Z. Ernährungsw. 5,* 174–183.
32. Ullrich, I.H., Lai, H.-Y., Vona, L., Reid, R.L., and Albrink, M.J. (1981) *Am. J. Clin. Nutr. 34,* 2054–2060.
33. Wells, W.W. (1957) *Arch. Biochem. Biophys. 66,* 217–225.
34. Cohen, B.I., Raicht, R.F., and Mosbach, E.H. (1974) *Lipids 9,* 1024–1029.

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Fatty Acid Allene Oxides. III. Albumin-induced Cyclization of 12,13(*S*)-Epoxy-9(*Z*),11-octadecadienoic Acid[1]

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Recently, corn *(Zea mays L.)* hydroperoxide dehydrase was found to catalyze the conversion of 13*(S)*-hydroperoxy-9*(Z)*,11*(E)*-octadecadienoic acid into an unstable fatty acid allene oxide, 12,13*(S)*-epoxy-9*(Z)*,11-octadecadienoic acid. This study is concerned with the chemistry of 12,13*(S)*-epoxy-9*(Z)*,11-octadecadienoic acid in the presence of vertebrate serum albumins.

Albumins were found to greatly enhance the aqueous half-life of the allene oxide, i.e. 14.1 ± 1.8 min, 11.6 ± 1.2 min and 4.8 ± 0.5 min at 0 C in the presence of 15 mg/ml of bovine, human and equine serum albumins, respectively, as compared with ca. 33 sec in the absence of albumin. Degradation of allene oxide in the presence of bovine serum albumin led to the formation of a novel cyclization product, i.e. 3-oxo-2-pentyl-cyclopent-4-en-1-octanoic acid (12-oxo-10-phytoenoic acid, in which the relative configuration of the side chains attached to the five-membered ring is *trans*). Steric analysis of the cyclic derivative showed that the compound was largely racemic (ratio between enantiomers, 58:42).

12-Oxo-10,15*(Z)*-phytodienoic acid, needed for reference purposes, was prepared by incubation of 13*(S)*-hydroperoxy-9*(Z)*,11*(E)*,15*(Z)*-octadecatrienoic acid with corn hydroperoxide dehydrase. Steric analysis showed that the 12-oxo-10,15*(Z)*-phytodienoic acid thus obtained was not optically pure but a mixture of enantiomers in a ratio of 82:18.

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In recent work, a fatty acid allene oxide, 12,13*(S)*-epoxy-9*(Z)*,11-octadecadienoic acid, was identified as the product formed from 13*(S)*-hydroperoxy-9*(Z)*,11*(E)*-octadecadienoic acid in the presence of corn hydroperoxide dehydrase (previously referred to as corn hydroperoxide isomerase) (1). The allene oxide was rapidly hydrolyzed ($t_{1/2}$ about 30 sec at 0 C) in aqueous medium into the previously recognized stable α-ketol, 12-oxo-13-hydroxy-9*(Z)*-octadecenoic acid. Treatment of allene oxide with an excess of methanol led to the formation of a trapping product, 12-oxo-13-methoxy-9*(Z)*-octadecenoic acid. Instability of the allene oxide was not confined to hydroxylic solvents. Thus, in acetonitrile solution the allene oxide structure was subject to intramolecular attack by the carboxyl group to yield two isomeric macrolactones, 12-oxo-9*(Z)*-octadecen-11-olide and 12-oxo-9*(Z)*-octadecen-13-olide (2).

Unstable eicosanoids like thromboxane A_2, prostaglandin I_2 and leukotriene A_4 are stabilized in the presence of vertebrate serum albumins (3–5). The aim of this work was to examine whether 12,13*(S)*-epoxy-9*(Z)*,11-octadecadienoic acid was stabilized in the same way. In the course of the investigation, a novel cyclization product formed from 12,13*(S)*-epoxy-9*(Z)*,11-octadecadienoic acid in the presence of bovine serum albumin was isolated and characterized.

EXPERIMENTAL PROCEDURES

Materials. 13*(S)*-hydroperoxy-9*(Z)*,11*(E)*-octadecadienoic acid (13*(S)*-[1-^{14}C]HPOD) (specific radioactivity, 37 and 1.4 kBq/μmol) and 13*(S)*-hydroperoxy-9*(Z)*,11*(E)*,15*(Z)*-octadecatrienoic acid (13*(S)*-[1-^{14}C]HPOT) (specific radioactivity, 1.5 kBq/μmol) were prepared by incubation of [1-^{14}C]-linoleic and [1-^{14}C]α-linolenic acids, respectively, with soybean lipoxygenase as previously described (6). 13*(S)*-[9,10,12,13-$^2H_4$]-HPOD was prepared from [9,10,12,13-$^2H_4$]linoleic acid as described (1). Crystalline, fatty acid-free (less than 0.005%) serum albumins were purchased from Sigma Chemical Co. (St. Louis, MO).

Enzyme preparation. A preparation containing corn hydroperoxide dehydrase was obtained by extraction of defatted corn germ with potassium phosphate buffer followed by ammonium sulfate precipitation (0–45% saturation) as described (1). Precipitate dissolved in ice-cold 0.1 M potassium phosphate buffer, pH 7.4 (1.6 mg of protein per ml buffer) was used for the incubations.

Methyl 12-oxo-10,15(Z)*-phytodienoate* (1). 13*(S)*-[1-^{14}C]-HPOT (8.4 mg) was dissolved in 0.5 ml of acetonitrile and shaken with 45 ml of enzyme preparation (72 mg of protein) at 0 C for 15 min. Methanol (3 vol.) was added, and the mixture was carefully acidified to pH 3 and extracted with two portions of diethyl ether. The product (recovery of radioactivity, 93%) was treated with diazomethane and subjected to thin layer chromatography (TLC) (solvent system, ethyl acetate/hexane [15:85, v/v]). A broad peak of radioactivity appeared (about 80% of the radioactivity applied, R_f = 0.26–0.36). This material was subjected to reversed-phase high performance liquid chromatography (HPLC). Three peaks of radioactivity appeared, i.e. methyl 12-oxo-13-hydroxy-9*(Z)*,15*(Z)*-octadecadienoate (67%; 18.6–20.2 ml effluent), methyl 13*(S)*-hydroxy-9*(Z)*,11*(E)*,15*(Z)*-octadecatrienoate (3%; 22.2–23.0 ml of effluent), and methyl 12-oxo-10,15*(Z)*-phytodienoate (30%; 23.0–24.6 ml of effluent). The last mentioned material was collected (yield, 1.3 mg). Its identity as methyl 12-oxo-10,15*(Z)*-phytodienoate (*1*, Fig. 1; see ref. 7 for original isolation and characterization of this compound) was based on 1) its UV spectrum, which showed λ_{max} at 221 nm (ε about 12,000), 2) its mass spectrum, which showed prominent ions at m/e 306 (M, 36%), 275 (M-31, 27), 238 (M-68 [β-cleavage with loss of C_5H_8], 47), 206 (238-32, 18), 177 (M-129 [loss of $\cdot(CH_2)_5$-$COOCH_3$], 41), 163 (M-143 [loss of $\cdot(CH_2)_6$-$COOCH_3$], 53), 149 (M-157 [loss of $\cdot(CH_2)_7$-$COOCH_3$], 49), 109 (65), 107 (42), 96 (100) and

[1]The first paper in this series is Reference 1.

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Abbreviations: 13*(S)*-HPOD, 13*(S)*-hydroperoxy-9*(Z)*,11*(E)*-octadecadienoic acid; 13*(S)*-HPOT, 13*(S)*-hydroperoxy-9*(Z)*,11*(E)*,15*(Z)*-octadecatrienoic acid; MC, (-)-menthoxycarbonyl, Me_3Si, trimethylsilyl; Phytonoic acid, 2-pentyl-cyclopentane-1-octanoic acid, in which the two side chains have the *cis* relationship (7,8); Phytonoic acid (*trans* isomer), 2-pentyl-cyclopentane-1-octanoic acid, in which the two side chains have the *trans* relationship; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

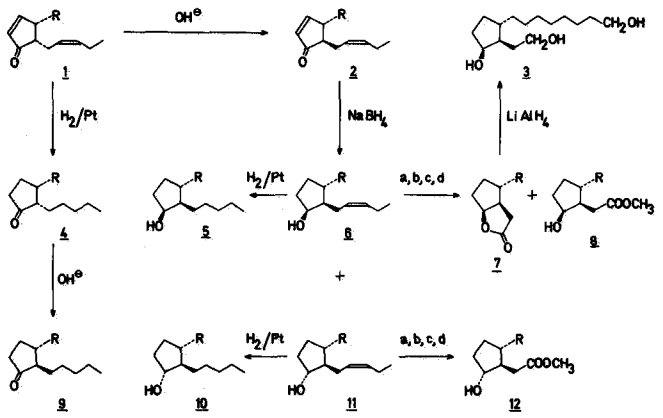

FIG. 1. Structures of methyl 12-oxo-10,15(*Z*)-phytodienoate *(1)* and derivatives. *1* is shown as the 9*(S)*,13*(S)* enantiomer in order to conform with ref. 8, although the absolute configuration of *1* has not been published. a, Acetylation by acetic anhydride/pyridine; b, oxidative ozonolysis; c, alkaline hydrolysis; d, esterification by treatment with diazomethane. R, $(CH_2)_7$-$COOCH_3$.

82 (49), and 3) its conversion into two epimeric 12-hydroxy-15*(Z)*-phytoenoates upon sodium borohydride reduction (8, and below).

Thin layer chromatographic analysis (solvent, ethyl acetate/hexane [2:8, v/v]) of the methyl ester of 12-oxophytodienoic acid obtained in this way showed the presence of a major (94%; R_f = 0.42) and a minor (6%; R_f = 0.47) isomer. The major isomer was due to *1*, in which the side chains attached to C-9 and C-13 had the *cis* relationship, whereas the latter isomer was the corresponding *trans* derivative *2*. These assignments follow from the work by Vick and Zimmerman, who showed that 12-oxophytodienoic acid biosynthesized from 13*(S)*-HPOT was the *cis* isomer (8) and from the fact that *1* prepared as described above was, as expected (8–11), rapidly isomerized into the more stable *trans* form *(2)* by base treatment (see below). Gas liquid chromatographic analysis of *1* showed a major peak at C 24.40 and a minor peak at C 24.00 corresponding to *1* and *2*, respectively. Such thermal isomerization of *cis* phytonoate derivatives during GLC has been reported (8).

Methyl 12-Oxo-10,15(Z)*-phytodienoate (*trans *isomer)* (2). *1* (1 mg) was treated with 0.1 M sodium hydroxide in 90% aqueous methanol at room temperature for one hr and subsequently extracted and treated with diazomethane. Following preparative TLC, about 0.8 mg of pure *2* was obtained. The ultraviolet spectrum of *2* showed an absorption band with λ_{max} at 222 nm (ε about 12,000). Gas liquid chromatographic analysis showed a single peak (C 24.00).

*Methyl 12-Oxophytonoate (*cis *and* trans *isomers) (4 and 9). 1* (1 mg) was subjected to catalytic hydrogenation, and the product was subjected to preparative TLC. In this way, about 0.7 mg of *4* was obtained. GLC showed a major peak at C-23.60 *(4)* and a minor peak at C-23.25 (due to *9* formed by thermal isomerization) (Fig. 2a). Treatment of *4* (0.5 mg) with 5 ml of 0.5 M NaOH in 50% aqueous methanol at 37 C for 18 hr followed by esterification resulted in the formation of a mixture of *9* (C 23.25) and *4* (C 23.60) in a ratio of about 92:8 (Fig. 2b). Alternatively, pure *9* could be prepared by catalytic hydrogenation

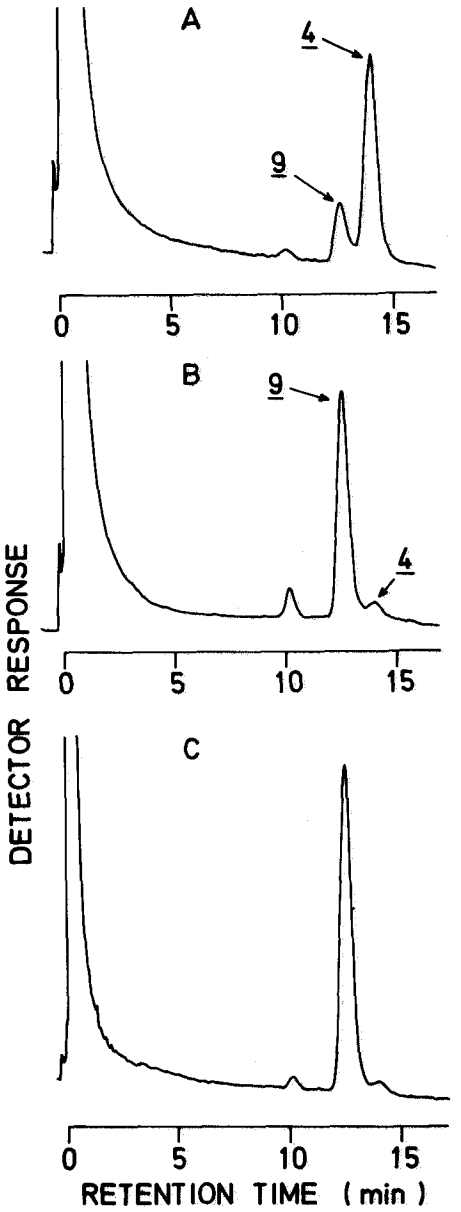

FIG. 2. GLC analysis of methyl 12-oxophytonoates. Column, 5% QF-1 on Supelcoport; column temperature, 190 C; injection port temperature, 220 C. (A) Injection of *4*. Thermal isomerization into *9* is apparent. (B) Injection of *9* (containing ca. 8% of *4*). (C) Injection of methyl 12-oxophytonoate obtained by catalytic hydrogenation of Compound B.

of *2*. No separation of *4* and *9* was observed on TLC analysis (solvent system, ethyl acetate/hexane (2:8, v/v); R_f = 0.68).

The mass spectrum of *9* showed prominent ions at m/e 279 (M-31, 4%), 240 (M-70 [β-cleavage with loss of CH_2=CH-C_3H_7], 14), 153 (M-157 [loss of ·$(CH_2)_7$-$COOCH_3$], 22), 83 (100) and 82 (50).

Methyl 12-hydroxy-15(Z)*-phytoenoates (*trans *isomers, two epimers) (6 and* 11*). 2* (1 mg) was treated with sodium borohydride, and the product was subjected to TLC (solvent, ethyl acetate/hexane (25:75, v/v)). Two compounds appeared, i.e. *6* (39%; R_f = 0.59) and *11* (61%; R_f = 0.48). The mass spectrum of the Me_3Si derivative of *11* showed

prominent ions at m/e 382 (M, 1%), 367 (M-15, 1), 351 (M-31, 1), 311 (M-71 [loss of $\cdot C_5H_{11}$], 2), 292 (M-90 [loss of Me_3SiOH], 5), 249 (3), 225 (M-157 [loss of $\cdot(CH_2)_7$-$COOCH_3$], 100) and 135 (225-90, 22). The mass spectrum of the trimethylsilyl (Me_3Si) derivative of *6* was similar to that of *11* although, in this case, the ion m/e 135 (225-90) was the base peak.

To examine the relative configuration of the alcohol group at C-12 and the pentyl side chain at C-13 of *6* and *11*, the following experiment was carried out (Fig. 1). *6* and *11* (0.5 mg) were separately acetylated and subjected to oxidative ozonolysis. The products were treated with 1 M NaOH in 80% aqueous methanol at room temperature for 18 hr. The hydrolysis mixtures were acidified to pH 1 and extracted with two portions of ethyl acetate. Analysis of the esterified products by TLC (solvent, ethyl acetate/hexane [25:75, v/v]) demonstrated the formation of diester *12* (R_f = 0.20) from *11*, whereas the product obtained from *6* contained lactone *7* (R_f = 0.34) and diester *8* (R_f = 0.22) in a ratio of about 2:1. The structures of diesters *8* and *12* were confirmed by mass spectrometry (Me_3Si derivatives; prominent ions at m/e 371 (M-15, 23%), 355 (M-31, 1), 339 (M-[15+32], 3), 313 (M-73 [loss of $\cdot CH_2$-$COOCH_3$], 8), 312 (M-74 [loss of CH_2=C(OH)OCH_3], 13), 155 (M-[74+157] [loss of CH_2=C(OH)OCH_3 plus $\cdot(CH_2)_7$-$COOCH_3$], 100), and 129 (CH_2=CH-CH=O^+SiMe_3, 26). The structure of lactone *7* was established by its mass spectrum, which showed a molecular ion at m/e 282, its conversion into *8* by alkaline hydrolysis followed by esterification, and its conversion into triol *3* by $LiAlH_4$ reduction.

It followed from the above conversions that *6* had the *cis* relationship between the C-12 alcohol group and the C-13 side chain, while in *11* the relationship between the corresponding substituents was *trans*.

*Methyl 12-hydroxyphytonoates (*trans *isomers, two epimers) (*5 *and* 10*). 5* and *10* were prepared by catalytic hydrogenation of *6* and *11*, respectively. The R_f-values found on TLC analysis were 0.58 *(5)* and 0.51 *(10)* (solvent, ethyl acetate/hexane [25:75, v/v]), and the C-values found on GLC analysis of the Me_3Si derivatives were 20.00 *(5)* and 19.85 *(10)*. The mass spectra of the two derivatives were virtually identical and showed prominent ions at m/e 384 (M, 1%), 369 (M-15, 15), 337 (M-[15+32], 9), 294 (M-90, 10), 137 (M-[157+90] [loss of $\cdot(CH_2)_7$-$COOCH_3$ plus Me_3SiOH], 7) and 129 (CH_2=CH-CH=O^+SiMe_3, 100).

Methods for isolation and structure determination. TLC was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck (Darmstadt, FRG). Material was located by spraying with 2′,7′-dichlorofluorescein and viewing under UV light. HPLC was performed using a column (300 × 8 mm) of Polygosil C_{18} 5 μ and acetonitrile/water (85:15, v/v) at a flow rate of 1.5 ml/min. The absorbancy of the effluent at 222 nm was measured. GLC was performed with a F&M Biomedical gas chromatograph model 402 using a column of 5% QF-1 on Supelcoport. Gas chromatography-mass spectrometry (GC-MS) was carried out with an LKB 9000S instrument. The electron energy was set at 22.5 eV, and the trap current was 60 μA. Ultraviolet spectra were recorded with a Hewlett-Packard model 8450A UV/VIS spectrophotometer. Radioactivity was determined with a Packard Tri-Carb series 4000 liquid scintillation counter. A Berthold Dünnschichtscanner II was used for determination of radioactivity on TLC plates.

Sodium borohydride reduction of carbonyl compounds (0.1–2 mg) was carried out at room temperature for 30 min with 20 mg of $NaBH_4$ in 2 ml of methanol. $LiAlH_4$ reduction was performed by refluxing the sample (0.1–1 mg) for two hr with 70 mg of $LiAlH_4$ in 15 ml of diethyl ether. Methods for catalytic hydrogenation (12), oxidative ozonolysis (13), and preparation of Me_3Si (12) and *O*-methyloxime derivatives (1) were as described.

*Method for measurement of aqueous half-life of 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid in the presence of albumins.* 13*(S)*-[1-^{14}C]HPOD (90 nmol, 3.3 kBq) in 50 μl of acetonitrile was stirred with 7 ml of enzyme preparation for 20 sec at 0 C. One ml of serum albumin solution in potassium phosphate buffer was added (final concentration of albumin, 15 mg/ml), and stirring at 0 C continued. Aliquots of 1 ml were removed 2, 4, 8, 15, 25 and 40 min after addition of serum albumin. In the case of egg albumin, the times used were 20, 40, 60, 80 sec and 2, 5, 10 and 15 min. Samples were treated with 20 ml of methanol and kept for one hr at room temperature. [1-^{14}C]Oleic acid (152 Bq) was added as an internal standard, and the mixtures were extracted with diethyl ether. The esterified materials were subjected to radio-TLC (solvent, ethyl acetate/hexane [2:8, v/v]). The peaks corresponding to radioactive methyl 12-oxo-13-methoxy-9*(Z)*-octadecenoate (R_f = 0.69; formed from allene oxide in the presence of methanol) and methyl oleate (R_f = 0.84) were cut out and weighed. The ratios determined for the different time points were plotted on a logarithmic scale vs time, and the half-life times were calculated.

RESULTS AND DISCUSSION

Stabilization of allene oxide by serum albumins. As seen in Figure 3, degradation of 12,13*(S)*-epoxy-9*(Z)*,11-octadecadienoic acid in aqueous medium followed first-order kinetics. The half-life time in the absence of albumin was ca. 33 sec (1). In the presence of different serum albumins, the half-life was increased by a factor of 9–26. On the other hand, egg albumin, which is unrelated to vertebrate serum albumins, did not stabilize the allene oxide. As seen in Table 1, the efficacy of stabilizing allene oxide by the different serum albumins tested was bovine > human > equine. Interestingly, the same ranking order was found for these albumins in stabilizing leukotriene A_4 (5). It seems likely that stabilization occurs by binding of the unstable compound to hydrophobic binding sites of the albumin molecule. Studies are in progress to examine the possible role of specific binding sites I and II (14) in stabilizing allene oxide.

Isolation and structure of Compound B. During studies of the half-life of allene oxide in the presence of bovine serum albumin described above, an unknown compound was observed with an R_f-value intermediate to those of methyl 12-oxo-13-hydroxy-9*(Z)*-octadecenoate and the methyl ester of 13*(S)*-HPOD. As seen in Figure 4A, the yield of the new compound (B) was maximal (24% of the recovered product) when allene oxide generated from 13*(S)*-HPOD was treated for one hr at 0 C with 15 mg/ml of bovine serum albumin before quenching with methanol. When the time of treatment with albumin was reduced to 15 min, the yield of Compound B dropped to 8%. Also, formation of 12-oxo-13-hydroxyoctadecenoate was some-

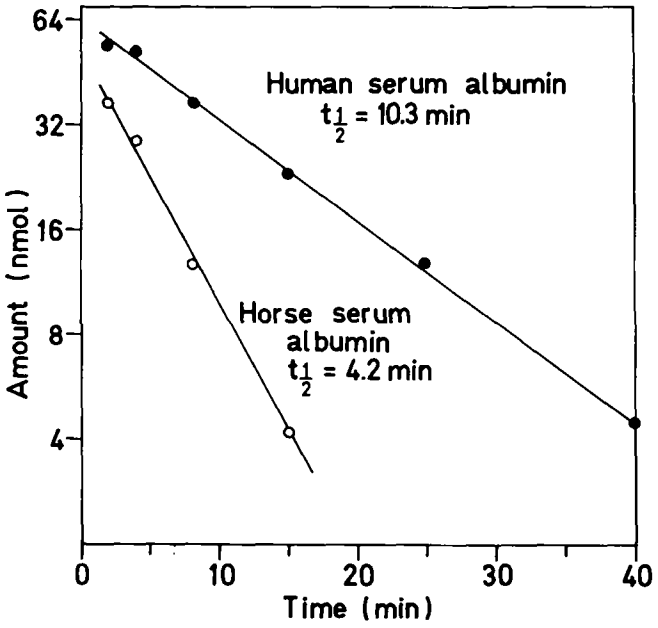

FIG. 3. Degradation of 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid in the presence of human serum albumin (15 mg/ml; – ● – ● – ● –) and horse serum albumin (15 mg/ml; —O—O—O—).

TABLE 1

Stabilization of 12,13(*S*)-Epoxy-9(*Z*),11-octadecadienoic Acid by Albumins (15 mg/ml)

Albumin	Half-life of allene oxide, mean ± S.D. (min)
—	0.55[a]
Bovine	14.1 ± 1.8 (n = 3)
Human	11.6 ± 1.2 (n = 3)
Equine	4.8 ± 0.5 (n = 3)
Chicken egg	0.59 ± 0.02[b] (n = 3)

[a]Ref. 1.

[b]Initial phase, during which 80% of allene oxide was hydrolyzed into α-ketol.

what reduced. Instead, an increased yield of methyl 12-oxo-13-methoxy-9(*Z*)-octadecenoate was noticed (Fig. 4B). If the albumin treatment was omitted, i.e. the allene oxide generated was directly treated with methanol, Compound B was not detectable, and the yield of methyl 12-oxo-13-methoxyoctadecenoate was further increased (Fig. 4C). Additionally, when allene oxide was allowed to hydrolyze into 12-oxo-13-hydroxy-9(*Z*)-octadecenoic acid before addition of albumin, only methyl 12-oxo-13-hydroxyoctadecenoate was observed (Fig. 4D). These experiments thus demonstrated an albumin- and time-dependent formation of Compound B from 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid.

Larger amounts of Compound B were prepared in the following way: 13(*S*)-[1-^{14}C]HPOD, 3.3 mg, was stirred with 42 ml of enzyme preparation at 0 C for 30 sec. A solution of 675 mg of fatty acid-free bovine serum albumin in 3 ml of potassium phosphate buffer was added, and

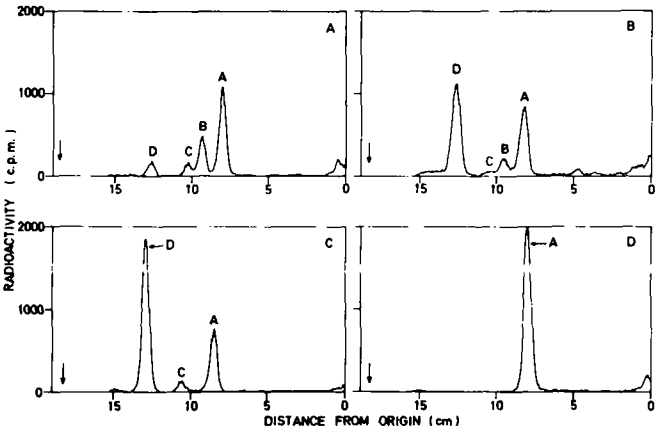

FIG. 4. Thin layer radiochromatographic analysis of products formed upon incubation of 13(*S*)-[1-^{14}C]HPOD. (A) Consecutive treatments were as follows: 1) stirring of 90 nmol of 13(*S*)-HPOD with 5 ml of enzyme preparation for 10 sec at 0 C; 2) addition of 1 ml of solution of bovine serum albumin (final concentration, 15 mg/ml) and stirring for 60 min at 0 C; 3) addition of 60 ml of methanol. (B) As in A, but time of treatment with albumin was 15 min. (C) Consecutive treatments as follows: 1) stirring of 90 nmol of 13(*S*)-HPOD with 5 ml of enzyme preparation for 10 sec at 0 C; 2) addition of 50 ml of methanol. (D) As in A, but time of treatment with enzyme preparation was five min.

Peak A, methyl 12-oxo-13-hydroxy-9(*Z*)-octadecenoate; peak B, Compound B; peak C, methyl 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate; peak D, methyl 12-oxo-13-methoxy-9(*Z*)-octadecenoate. Solvent system, ethyl acetate/hexane (2:8, v/v). Solvent front is indicated by arrow.

the mixture was stirred at 0 C for 60 min. The incubation mixture was added to 135 ml of methanol and kept at room temperature for 30 min. The product obtained by diethyl ether extraction (recovery of radioactivity, about 85%) was treated with diazomethane and subjected to TLC (Fig. 4). Compound B obtained in this way was radiochemically pure. Traces of unlabeled impurities were removed by reversed-phase HPLC (Compound B eluted with an effluent volume of 28.2–30.1 ml).

The mass spectrum of Compound B is given in Figure 5. As seen, prominent ions appeared at m/e 308 (M, 3%), 277 (M-31, 13), 238 (M-70 [β-cleavage with loss of CH_2=CH-C_3H_7], 38), 206 (238-32, 22), 178 (25), 165 (M-143 [loss of ·$(CH_2)_6$-$COOCH_3$], 12), 151 (M-157 [loss of ·$(CH_2)_7$-$COOCH_3$], 30), 109 (46), 96 (100), and 82 (49). The similarity of this spectrum with that of *1* suggested that Compound B was a phytonoate derivative. The molecular ion at m/e 308 indicated the presence of one double bond. This double bond had to be located in the five-membered ring because the ions at m/e 238 and 151 showed that the side chains attached to C-13 and C-9, respectively, were both saturated.

Conclusive evidence for the presence of a cyclopentenone structure in Compound B was provided by ultraviolet spectrometry, which showed an absorption band with λ_{max} at 222 nm (ε about 12,000).

The mass spectrum of the *O*-methyloxime derivative of Compound B showed prominent ions at m/e 337 (M, 51%), 306 (M-31, 100), 274 (M-[31+32], 46), 267 (M-70 [β-cleavage with loss of CH_2=CH-C_3H_7], 77), 236 (267-31, 95), 194 (M-143 [probably loss of ·$(CH_2)_6$-$COOCH_3$], 43), and 124 (194-70, 53).

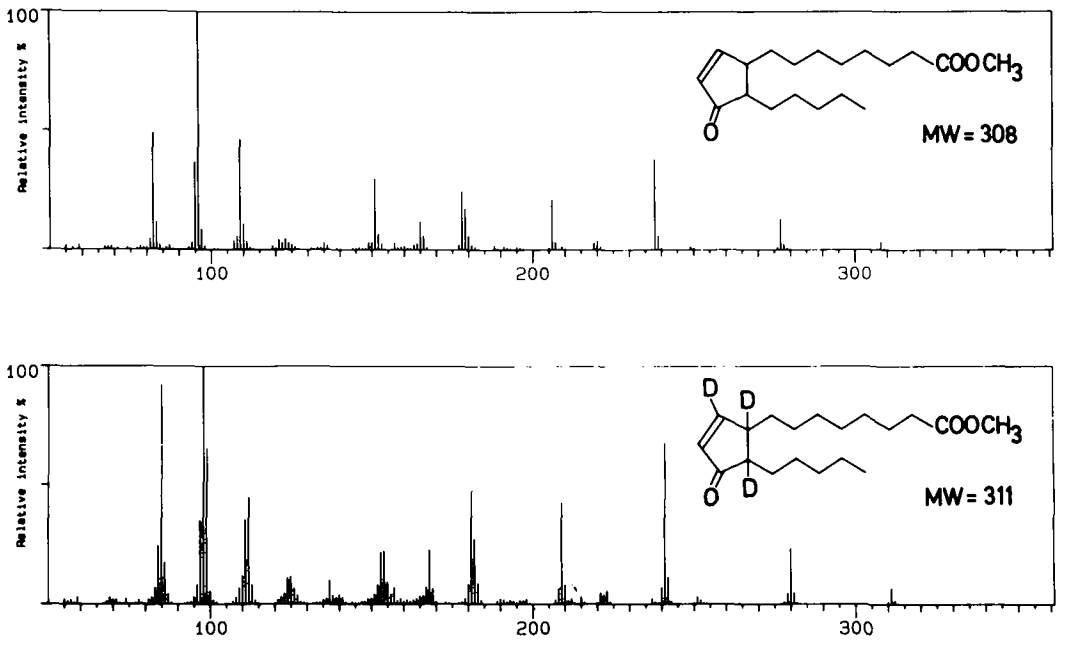

FIG. 5. Mass spectra of Compound B (upper) and of Compound B obtained following incubation of 13*(S)*-[9,10,12,13-$^2H_4$]-HPOD (lower).

Treatment of Compound B with 0.5 M NaOH in 50% aqueous methanol at 70 C for one hr resulted in smooth conversion into an isomeric derivative having λ_{max} at 238 nm (ε about 16,000). The mass spectrum of the methyl ester of this compound (C-value, 24.80) showed prominent ions at m/e 308 (M, 14%), 277 (M-31, 10), 179 (M-129 [elimination of $\cdot(CH_2)_5$-$COOCH_3$ by cleavage between C-6 and C-7], 92), 166 (M-142 [elimination of CH_2=CH-$(CH_2)_4$-$COOCH_3$ by cleavage between C-7 and C-8 and transfer of 1 H], 8), 151 (M-157 [elimination of $\cdot(CH_2)_7$-$COOCH_3$], 100), 123 (14) and 110 (21). This fragmentation pattern was similar to those reported for methyl 12-oxo-9(13),15*(Z)*-phytodienoate (8) and 2-heptyl-3-(7-carbomethoxyheptyl)-cyclopent-2-enone (15). On the basis of the data mentioned, the derivative obtained by treatment of Compound B with sodium hydroxide was assigned the structure 12-oxo-9-13)-phytoenoic acid (free acid of *13*, see Fig. 6).

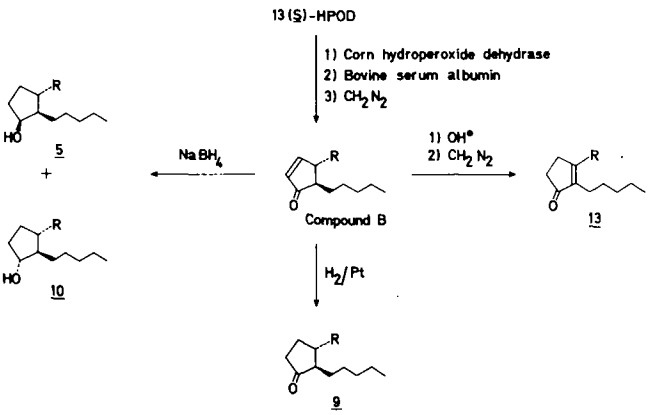

FIG. 6. Chemical conversions carried out on Compound B (shown as the 9*(S)*,13*(R)* enantiomer). R, $(CH_2)_7$-$COOCH_3$.

Catalytic hydrogenation of Compound B afforded a dihydro derivative that was identical to a methyl 12-oxophytonoate as judged by TLC and mass spectrometry (reference compounds: *4* and *9*). The retention time of the major peak of hydrogenated Compound B was identical with that of *9* although a minor peak (less than 5%) appeared at the position of *4* (Fig. 2). Thus, the two side chains attached to the ring structure of Compound B had mainly (>95%) the *trans* relationship. Phytonoic and jasmonic acid derivatives in which the relative configuration of the two side chains is *cis* are readily isomerized into the more stable *trans* derivatives by acid, base, or heat (8–11). Thus, it was conceivable that Compound B initially formed from the allene oxide had the *cis* relationship between the side chains but underwent isomerization into the *trans* compound during the isolation procedures. Also, interaction of Compound B with a hydrophobic alkaline microenvironment of albumin (5) possibly would promote such an isomerization. Incubations of 13*(S)*-[9,10,12,13-$^2H_4$]-HPOD were carried out to determine whether Compound B was initially formed as the *cis* or *trans* derivative. Analysis of Compound B by mass spectrometry revealed the presence of 91% trideuterated and 9% dideuterated molecules (Fig. 5). Because the deuterated hydroperoxide incubated consisted of 93% tetradeuterated and 7% trideuterated molecules, it was apparent that Compound B almost completely retained 3 deuteriums (at C-9, C-10, and C-13) and, as expected, lost the C-12 deuterium. Retention of the C-13 deuterium atom meant that no appreciable exchange of hydrogen/deuterium occurred at this carbon, neither during the incubation period with albumin nor during the isolation procedures. Consequently, Compound B formed from 12,13*(S)*-epoxy-9*(Z)*,11-octadecadienoic acid in the presence of albumin had the *trans* relationship between its two side chains.

Sodium borohydride reduction of Compound B afforded two epimeric methyl 12-hydroxyphytonoates. The two epimers were formed in a ratio of 4:6 and were found to be identical with *5* and *10,* respectively, as judged by analysis by TLC, GLC and mass spectrometry (Fig. 6).

On the basis of these data, Compound B was assigned the structure methyl 3-oxo-2-pentyl-cyclopent-4-en-1-octanoate, i.e. the methyl ester of 12-oxo-10-phytoenoic acid (*trans* isomer) (Fig. 8).

Enantiomeric composition of Compound B. 5 and *10,* prepared from Compound B (above and Fig. 6), were separately treated with (−)-menthylchloroformate (13), and the resulting (−)-menthoxycarbonyl (MC) derivatives were purified by TLC (solvent system, ethyl acetate/hexane [7:93, v/v]). Each of the MC derivatives gave two peaks of diastereomers when analyzed by GLC. The two diastereomers of *5*-MC appeared in a ratio of 58:42 (faster to slower eluting diastereomer, respectively; separation factor, 1.08) (Fig. 7A). As expected, the two diastereomers of *10*-MC appeared in similar proportions (59:41, slower to faster eluting derivative, respectively; separation factor, 1.05). Thus, Compound B formed from 12,13*(S)*-epoxy-9*(Z)*,11-octadecadienoic acid essentially was racemic, which is in agreement with its formation by a nonenzymatic, albumin-promoted reaction.

Enantiomeric composition of 1. The methyl ester of 12-oxo-10,15*(Z)*-phytodienoic acid *(1)* was converted into *5* and *10* by successive treatments with sodium hydroxide, sodium borohydride, and hydrogen gas (Fig. 1). The MC derivatives of *5* and *10* were prepared and analyzed by GLC as described above for Compound B. The MC derivative of *5* separated into two diastereomers in a ratio of 82:18 (slower to faster eluting diastereomer, respectively; separation factor, 1.08) (Fig. 7B). Two diastereomers were observed upon GLC analysis of *10*-MC. The ratio found was 78:22 (faster to slower eluting isomer). However, due to the small separation factor (1.05), the percentage of the minor, slower eluting diastereomer probably was overestimated.

Vick and Zimmerman have provided evidence that 12-oxo-10,15*(Z)*-phytodienoic acid serves as the precursor of (+)-7-iso-jasmonic acid in plant tissue (16). In that context, it was notable that 12-oxo-10,15*(Z)*-phytodienoic acid biosynthesized from 13*(S)*-HPOT in the presence of corn hydroperoxide dehydrase was found not to be optically pure but a mixture of enantiomers in a 82:18 ratio. The absolute configurations of the major and minor enantiomers of *1* and of Compound B recently have been determined (Hamberg, M., Miersch, O., and Sembdner, G., unpublished data).

This study demonstrates that 12,13*(S)*-epoxy-9*(Z)*,11-octadecadienoic acid, a fatty acid allene oxide derived from 13*(S)*-HPOD, is strongly stabilized by serum albumins. Stabilization was greatest with bovine serum albumin and probably was due to binding of allene oxide to hydrophobic binding sites of the albumin molecule.

Degradation of allene oxide in the presence of bovine serum albumin led to the formation of a cyclopentenone derivative, i.e. essentially racemic 12-oxo-10-phytoenoic acid (*trans* isomer) (Fig. 8). In this context, previous studies in which cyclopentenones have been isolated following peracid epoxidation of vinylallene derivatives should be mentioned (17,18). Allene oxides and/or cyclopropanones were suggested as intermediates in those conversions.

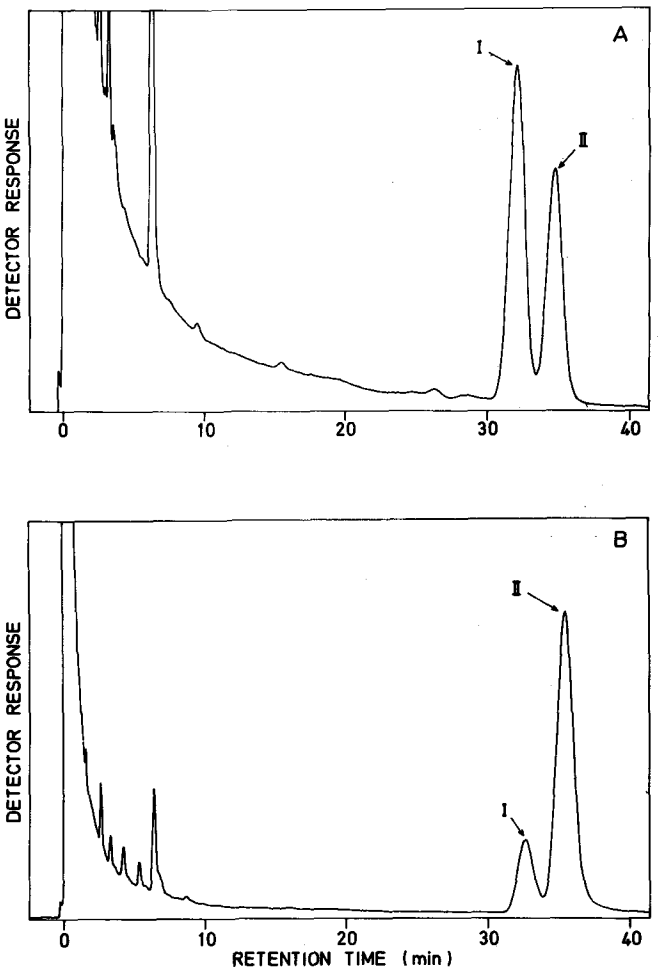

FIG. 7. Steric analysis of MC derivative of *5* derived from Compound B (A) and from methyl 12-oxo-10,15*(Z)*-phytodienoate (B). Column, 5% QF-1; column temperature, 220 C.

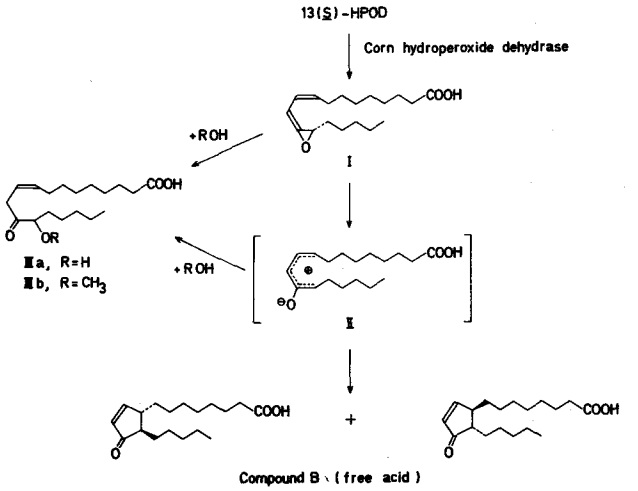

FIG. 8. Cyclization of allene oxide generated from 13*(S)*-HPOD. I, 12,13*(S)*-epoxy-9*(Z)*,11-octadecadienoic acid; II, dipolar ion formed from I by heterolysis of the bond between the epoxide oxygen and C-13; IIIa, 12-oxo-13-hydroxy-9*(Z)*-octadecenoic acid; IIIb, 12-oxo-13-methoxy-9*(Z)*-octadecenoic acid.

Our finding that an enzymatically generated fatty acid allene oxide can cyclize into a cyclopentenone derivative under suitable conditions suggests that an allene oxide is similarly involved as the precursor of 12-oxo-10,15*(Z)*-phytodienoic acid in different plant tissues. This proposal is supported by recent experiments that have shown that using conditions under which 12-oxophytodienoic acid is biosynthesized from 13*(S)*-HPOT, transient formation of 12,13*(S)*-epoxy-9*(Z)*,11,15*(Z)*-octadecatrienoic acid can be detected by chemical and physical methods (Hamberg, M., unpublished data).

ACKNOWLEDGMENTS

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REFERENCES

1. Hamberg, M. (1987) *Biochim. Biophys. Acta 920,* 76-84.
2. Hamberg, M.(1988) *Chem. Phys. Lipids 46,* 235-243.
3. Folco, G.C., Granström, E., and Kindahl, H. (1977) *FEBS Lett. 82,* 321-324.
4. Wynalda, M.A., and Fitzpatrick, F.A. (1980) *Prostaglandins 20,* 853-861.
5. Fitzpatrick, F.A., Morton, D.R., and Wynalda, M.A. (1982) *J. Biol. Chem. 257,* 4680-4683.
6. Hamberg, M., and Gotthammar, B. (1973) *Lipids 8,* 737-744.
7. Zimmerman, D.C., and Feng, P. (1978) *Lipids 13,* 313-316.
8. Vick, B.A., and Zimmerman, D.C. (1979) *Lipids 14,* 734-740.
9. Tanaka, H., and Torii, S. (1975) *J. Org. Chem. 40,* 462-465.
10. Fukui, H., Koshimizu, K., Yamazaki, Y., and Usuda, S. (1977) *Agric. Biol. Chem. 41,* 189-194.
11. Miersch, O., Preiss, A., Sembdner, G., and Schreiber, K. (1987) *Phytochemistry 26,* 1037-1039.
12. Hamberg, M., Herman, C.A., and Herman, R.P. (1986) *Biochim. Biophys. Acta 877,* 447-457.
13. Hamberg, M. (1971) *Anal. Biochem. 43,* 515-526.
14. Sudlow, G., Birkett, D.J., and Wade, D.N. (1976) *Mol. Pharmacol. 12,* 1052-1061.
15. Samuelsson, B., and Ställberg, G. (1963) *Acta Chem. Scand. 17,* 810-816.
16. Vick, B.A., and Zimmerman, D.C. (1983) *Biochem. Biophys. Res. Commun. 111,* 470-477.
17. Grimaldi, J., and Bertrand, M. (1969) *Tetrahedron Lett.,* 3269-3272.
18. Malacria, M., and Roumestant, M.L. (1977) *Tetrahedron 33,* 2813-2817.

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Compensatory Mechanisms in Erythrocyte Lipids in Patients with Atherosclerosis

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The quantitative composition of phospholipids and fatty acids of erythrocytes was investigated in patients with atherosclerosis. It was stated that the erythrocyte lipids of atherosclerotic patients contained smaller quantities of phosphatidylcholine and phosphatidylinositol, a significantly larger quantity of sphingomyelin, and higher sphingomyelin/phosphatidylcholine and cholesterol/phospholipid ratios. The existence of compensatory changes was stated, which was evident in the reduction of palmitic and stearic acids and the increase of linoleic and eicosatrienoic acids in erythrocyte phospholipids. These changes in fatty acid composition probably cause minimal changes in the membrane fluidity induced by an increased cholesterol/phospholipid and sphingomyelin/phosphatidylcholine ratios. This paper was the first evidence of occurrence of those changes in erythrocytes during spontaneous atherosclerosis in human.

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The maintenance of membrane fluidity in precisely determined limits is one of indispensable conditions of proper functioning of cells. The principal influence of the membrane fluidity is exerted by the composition of lipid fatty acids, the nature and quantitative composition of individual phospholipid classes, and the value of cholesterol/phospholipid and sphingomyelin/phosphatidylcholine ratios.

The increase of the unsaturation degree of fatty acids intensifies the membrane fluidity, while the increase of cholesterol/phospholipid and sphingomyelin/phosphatidylcholine ratios reduces it. In physiological conditions, the lipids of erythrocyte cell membrane are "fluid," i.e. the lateral mobility of lipids is relatively quick (1).

Cholesterol, an integral component of cell membranes, maintains a lipid bilayer in an "intermediate fluid" state, ensuring greater mobility of the acyl chains in the gel state and limiting it in the liquid-crystalline state (2–4). This so-called condensing effect plays an essential role in functioning of the membrane enzymes (5,6), in processes of a substance transport across the cell membrane (7–9), and in maintaining of adequate rheologic properties of erythrocytes (10,11). Erythrocytes do not possess an enzymatic apparatus necessary for the synthesis of phospholipids and cholesterol (12), and, due to this, the changes in the lipid composition of the plasma lipoproteins (13). An increase of the cholesterol/phospholipid ratio in the plasma lipoproteins and the erythrocyte cell membranes, the blood platelets, the liver cells and the endothelium cell membranes is one of the typical metabolic disorders in the course of atherosclerosis (12). Such changes, impairing the normal structure of cell membranes, may give rise to significant disorders in their functions.

In a previous paper (14), we examined the cytoprotective effect of prostaglandins on the erythrocytes of patients with atherosclerosis, stating slight differences in the osmotic resistance of erythrocytes and the lack of differences in the strength of this prostaglandin effect on healthy red blood cells and those in atherosclerotic patients.

The aim of this paper is to state whether there are any compensatory mechanisms in the erythrocytes of atherosclerotic patients, which would allow the integrity of the cell membrane in spite of the changes in the lipid composition induced by a disease.

MATERIALS AND METHODS

Blood taken from 24 male patients with atherosclerosis was used for estimations. The patients were divided into two groups: 15 patients on whom reconstruction of iliac and femoral arteries had been performed, and nine patients who had their limbs amputated at the thigh because of the extent of lesions. The control group (C) comprised of 11 males of the same age in whom detailed clinical examinations failed to demonstrate any disease and routine laboratory investigations gave normal results falling into the range accepted as not pathological.

In the fasting, serum of all patients and controls, the levels of triglycerides (TG), total cholesterol (Chol) and phospholipids (PL) were routinely determined using diagnostic test kits supplied by Boehringer (Mannheim, West Germany). High density lipoprotein (HDL) fraction of each serum also was obtained by the precipitation method using Boehringer kits, and the levels of the same components in this fraction were determined by the same methods as in whole serum as well. The type of dyslipidaemia in atherosclerotic patients was determined by electrophoretic separation of serum lipoprotines in agarose gel (15) and was found to be type IV according to Fredrickson (16) in all cases. Plasma and HDL lipid values of all patients are shown in Table 1.

All patients and controls fed a common nationwide Polish diet with an energetic value of 14693 kJ/person/daily. Animal fat consumption was 97 g/person/day, and the consumption of vegetable fat was only 25 g/person/day. Polyunsaturated fat intake did not exceeded 10% of the total (17). This diet remain unchanged within three months before experiment.

Lipids were extracted according to Bouhours and Bouhours (18). The lipid extract was dissolved in a small quantity of the chloroform/methanol (2:1, v/v) and phospholipid classes were separated by thin layer chromatography on the Silica Gel G plates (E. Merck, Darmstadt, West Germany). The neutral lipids were separated in solvent system: light petroleum/diethyl ether/acetic acid (90:10:1, v/v/v). Phospholipids were separated according

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Abbreviations: C, control group; Chol, cholesterol; HDL, high density lipoproteins; PL, phospholipids; TG, triglycerides.

TABLE 1

Levels of Triglycerides (TG), Cholesterol (Chol) and Phospholipids (PL) in Whole Plasma and High-density Lipoprotein (HDL) Fraction of Control Group (C) and in Patients with Atherosclerosis (I, II)

	Plasma mM/l			HDL fraction mM/l			
	TG	Chol	PL	TG	Chol	PL	
C	2.24 ± 0.25	6.04 ± 0.65	275 ± 34	0.36 ± 0.06	0.88 ± 0.10	42 ± 6	(11)
I	2.80 ± 0.32*	6.22 ± 0.70	333 ± 40*	0.52 ± 0.09*	0.80 ± 0.09	41 ± 5	(15)
II	2.94 ± 0.03*	6.16 ± 0.65	210 ± 24**	0.50 ± 0.07*	0.76 ± 0.09**	45 ± 6	(9)

In parentheses, the number of cases examined. Each result is the mean ± S.D.

*$p < 0.05$; Student's unpaired t-test.

**$p < 0.02$; Student's unpaired t-test.

to Stein and Smith (19). Chromatograms were visualized in iodine vapors, and compounds were identified by comparing their R_f values with those of standards (Sigma Chemical Co., St. Louis, MO). Individual spots were scraped off, and the gel was successively extracted with 5 ml each of chloroform, chloroform/methanol (2:1, v/v), chloroform/methanol (1:2, v/v) and methanol.

The pooled extracts were evaporated in vacuum, and cholesterol was determined in appropriate fractions according to Zlatkis et al. (20) and phosphorus as described by Bartlett (21).

In the parallel experiment, the zones containing phospholipid fractions were scraped off, extracted with 2 × 5 ml of the chloroform/methanol (1:1, v/v); the pooled extracts were evaporated in vacuum; the fatty acids were saponified with methanolic KOH (22). After acidification (1 M HCl) to pH = 2.0, the acids were extracted with light petroleum and turned into methyl esters with BF_3-methanol complex (Theodor Schuchardt, Hohenbrunn, West Germany) (23).

The composition and the quantity of fatty acids were determined by the gas chromatography with a Perkin Elmer F-30 gas chromatograph (Perkin Elmer Ltd., Beaconsfield, Bucks., England) as described (24). Identification of the components was carried out by comparing their retention times with those of standards (Applied Science Labs., State College, PA). Margaric acid was used as the internal standard. The peak fields and the retention times were measured by a computerized integrator System I (Spectra Physics, Santa Clara, CA). To resolute the components of very close retention times, the capillary column 50 m × 0.2 mm i.d., coated with CP Sil 88 also was used (Chrompack, Middelburg, Netherlands). The carrier gas was nitrogen, flow rate 0.5 mi/min, oven temperature from 220–270 C programmed with a rate of 1 C/min with flame ionization detector.

Student's unpaired t-test was used for comparing normally distributed data, and Wilcoxon's signed rank test for comparing data that normally were not distributed (25).

RESULTS

Table 2 shows the composition of phospholipids and sphingomyelin/phosphatidylcholine and cholesterol/phospholipid ratios in erythrocytes of healthy persons and those with atherosclerosis. It should be noted that there

TABLE 2

Erythrocyte Phospholipids of Control Group (C) and in Patients with Atherosclerosis (I and II)

	C (11)	I (15)	II (9)
PC	31.0 ± 1.3	27.2 ± 1.1*	26.8 ± 1.0[a]
PE	26.9 ± 1.6	25.8 ± 0.8	25.2 ± 1.0**
PS	12.6 ± 0.7	13.9 ± 0.7*	13.6 ± 1.1***
PI	4.9 ± 0.5	3.5 ± 0.4*	4.3 ± 0.6***
Sph	24.6 ± 2.1	29.6 ± 1.7*	30.1 ± 2.0*
Sph/PC	0.79 ± 0.07	1.09 ± 0.08*	1.12 ± 0.08*
Chol/PL	0.83 ± 0.07	1.64 ± 0.06*	1.67 ± 0.08*

In parentheses, the number of cases examined.

Each result is the mean ± S.D.

Composition as mol %.

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; Sph, sphingomyelin; Chol, cholesterol; PL, phospholipids.

*$p < 0.001$; Student's unpaired t-test.

**$p < 0.02$; Student's unpaired t-test.

***$p < 0.05$; Student's unpaired t-test.

was a significant reduction of the phosphatidylcholine quantity and increase of the phosphatidylserine and sphingomyelin quantities in erythrocytes of patients with atherosclerosis. The increase of cholesterol/phospholipid and sphingomyelin/phosphatidylcholine ratios in both groups also was significant. The differences in content of phospholipid fractions between both groups of atherosclerotic patients appeared not to be significant with exception of phosphatidylinositol.

Table 3 shows the fatty acid composition of the phosphatidylcholine fraction of erythrocytes. It can be observed that there is a considerable decrease of 16:0, 18:0 and 20:4ω6 acids, and a highly significant increase of 18:1ω9, 18:2ω6 and 20:3ω6 acids in the erythrocytes of patients with atherosclerosis.

Similar changes can be seen in fatty acid composition of phosphatidylethanolamine (Table 4) and phosphatidylinositol (Table 5). In the phosphatidylserine fraction (Table 6), the reduction of 16:0 and 18:0 is accompanied by the lowering of 20:4ω6 in group I, and its increase in group II to the level observed in the control. The sphingomyelin fraction of erythrocytes does not contain

TABLE 3

Fatty Acid Composition of Erythrocyte Phosphatidylcholine in Control Group (C), and in Patients with Atherosclerosis (I and II)

	C (11)	I (15)	II (9)
16:0	36.8 ± 2.4	30.1 ± 2.0*	28.2 ± 1.8*
18:0	11.8 ± 1.3	7.3 ± 0.8*	5.6 ± 0.5*
18:1ω9	17.4 ± 1.0	19.2 ± 0.8*	21.2 ± 0.8*
18:2ω6	21.5 ± 1.5	27.3 ± 1.6*	31.5 ± 1.9*
18:3ω3	0.7 ± 0.1	1.5 ± 0.3*	1.8 ± 0.4*
20:3ω6	2.0 ± 0.3	4.5 ± 0.4*	6.1 ± 0.5*
20:4ω6	5.1 ± 0.6	3.2 ± 0.5*	2.6 ± 0.5*
20:5ω3	0.4 ± 0.1	0.5 ± 0.1***	0.4 ± 0.1
22:4ω6	1.4 ± 0.2	2.3 ± 0.3**	1.5 ± 0.2
22:6ω3	2.9 ± 0.4	4.1 ± 0.4*	1.1 ± 0.1*

In parentheses, the number of cases examined.
Each result is the mean ± S.D.
Composition as mol %.
*$p < 0.001$; Student's unpaired t-test.
**$p < 0.02$; Student's unpaired t-test.
***$p < 0.05$; Student's unpaired t-test.

TABLE 4

Fatty Acid Composition of Erythrocyte Phosphatidylethanolamine in Control Group (C) and in Patients with Atherosclerosis (I and II)

	C (11)	I (15)	II (9)
16:0	21.6 ± 1.6	17.5 ± 1.3*	14.2 ± 1.0*
18:0	18.5 ± 1.0	13.2 ± 1.2*	11.3 ± 0.8*
18:1ω9	17.5 ± 0.9	19.3 ± 0.9*	22.1 ± 1.5*
18:2ω6	6.6 ± 0.7	11.5 ± 0.8*	13.6 ± 0.7*
18:3ω3	0.5 ± 0.1	1.1 ± 0.1*	2.1 ± 0.2*
20:3ω6	1.0 ± 0.1	2.2 ± 0.2*	3.0 ± 0.2*
20:4ω6	21.3 ± 2.3	18.4 ± 2.5**	15.4 ± 1.3*
20:5ω3	1.3 ± 0.3	3.2 ± 0.3*	4.4 ± 0.4*
22:4ω6	7.8 ± 1.1	8.5 ± 0.8	9.6 ± 0.8**
22:6ω3	3.9 ± 0.7	5.1 ± 0.6*	4.3 ± 0.3

In parentheses, the number of cases examined.
Each result is the mean ± S.D.
Composition as mol %.
*$p < 0.001$; Student's unpaired t-test.
**$p < 0.01$; Student's unpaired t-test.

fatty acids of the ω3 series (Table 7). Changes in acids of this fraction were found to be statistically not significant.

DISCUSSION

In this paper, we stated that the sphingomyelin and cholesterol quantities increase, and the phosphatidylcholine quantities in decrease in erythrocytes of atherosclerotic patients. These changes produce the increase of the sphingomyelin/phosphatidylcholine ratio similar to the phenomenon observed in abetalipoproteinaemia (26); the increase of the cholesterol/phospholipid ratio is similar to the increase in spur cell anemia (27). This leads to the reduction of cell membrane fluidity and reduces the

TABLE 5

Fatty Acid Composition of Erythrocyte Phosphatidylinositol in Control Group (C) and in Patients with Atherosclerosis (I and II)

	C (11)	I (15)	II (9)
16:0	5.3 ± 0.6	3.2 ± 0.5*	2.6 ± 0.4*
18:0	37.3 ± 2.2	32.1 ± 2.6*	29.3 ± 1.8*
18:1ω9	9.6 ± 1.2	10.5 ± 1.4	11.2 ± 1.5**
18:2ω6	4.3 ± 0.6	9.3 ± 0.5	12.3 ± 0.7*
18:3ω3	1.0 ± 0.2	2.1 ± 0.3*	2.5 ± 0.3*
20:3ω6	27.3 ± 3.0	32.1 ± 1.8*	34.2 ± 1.6*
20:4ω6	1.0 ± 0.2	0.5 ± 0.05*	0.4 ± 0.05*
20:5ω3	2.7 ± 0.4	1.6 ± 0.4*	1.5 ± 0.3*
22:4ω6	4.3 ± 0.5	5.3 ± 0.6*	4.8 ± 0.6
22:6ω3	7.2 ± 0.7	3.3 ± 0.4*	1.2 ± 0.3*

In parentheses, the number of cases examined.
Each result is the mean ± S.D.
Composition as mol %.
*$p < 0.001$; Student's unpaired t-test.
**$p < 0.05$; Student's unpaired t-test.

TABLE 6

Fatty Acid of Erythrocyte Phosphatidylserine in Control Group (C), and in Patients with Atherosclerosis (I and II)

	C (11)	I (15)	II (9)
16:0	4.0 ± 0.4	3.0 ± 04*	2.7 ± 0.3*
18:0	46.2 ± 2.6	40.1 ± 2.1*	38.3 ± 1.7*
18:1ω9	8.1 ± 1.1	6.8 ± 09**	7.2 ± 0.8
18:2ω6	3.2 ± 0.8	6.9 ± 0.6*	7.5 ± 0.7*
18:3ω3	1.1 ± 0.3	1.0 ± 0.3	1.2 ± 0.4
20:3ω6	24.2 ± 2.3	30.1 ± 2.4*	30.7 ± 2.5*
20:4ω6	0.2 ± 0.05	0.1 ± 0.05*	0.2 ± 0.05
20:5ω3	3.6 ± 0.5	2.0 ± 0.4*,[a]	3.1 ± 0.4 ***,[a]
22:4ω6	3.0 ± 0.4	4.6 ± 0.5**	4.7 ± 0.4**
22:6ω3	6.4 ± 0.9	5.4 ± 0.7**	4.4 ± 0.6*

In parentheses, the number of cases examined.
Each result is the mean ± S.D. Composition as mol %.
*$p < 0.001$; Student's unpaired t-test.
**$p < 0.01$; Student's unpaired t-test.
***$p < 0.05$; Student's unpaired t-test.
[a]Wilcoxon's signed rank test.

TABLE 7

Fatty Acid Composition of Erythrocyte Sphingomyelin in Control Group (C), and in Patients with Atherosclerosis (I and II)

	C (11)	I (15)	II (9)
16:0	34.2 ± 2.3	33.9 ± 2.2	34.4 ± 1.7
18:0	9.2 ± 0.6	9.4 ± 0.5	9.5 ± 0.4
18:1ω9	1.1 ± 0.3	1.3 ± 0.4	1.2 ± 0.5
20:0	3.8 ± 0.6	3.9 ± 0.5	3.8 ± 0.7
22:0	12.8 ± 1.4	13.4 ± 1.6	13.8 ± 1.7
24:0	24.2 ± 2.7	23.3 ± 2.5	24.5 ± 2.5
24:1ω6	3.3 ± 0.4	3.2 ± 0.5	3.1 ± 0.6
24:2ω6	11.4 ± 1.2	11.6 ± 0.9	9.7 ± 0.7

In parentheses, the number of cases examined.
Each result is the mean ± S.D.
Composition as mol %.

changes in contour of red blood cells (28). The increase of sphingomyelin and the decrease of phosphatidylcholine quantities in the course of atherosclerosis also were observed in the cells of aortal intima and media (29,30). Sphingomyelin stabilizes the bilayer structure of cell membranes (31), while too high cholesterol content destabilizes it (32); the increase of sphingomyelin quantity can be treated as the compensatory change in response to the increase of cholesterol.

The fluidity of cell membranes also depends on the fatty acid composition and their degree of unsaturation (33,34). It generally is accepted that unsaturated fatty acids intensify the membrane fluidity while saturated ones reduce it (12). The composition of acids of the plasma phospholipids in the course of atherosclerosis undergoes changes characterized by the increase in 16:0, 18:0 and 18:1ω9 (35); similar changes in the phosphatidylcholine fraction of the atherosclerotic aorta were noted by Böttcher and Van Gent (30).

Dietary manipulation also produces the changes in the composition of cell membrane fatty acids. Brasitus et al. (36) asserted that feeding a diet containing large amounts of 18:2ω6 increases the lipid fluidity and the unsaturation degree of acyl chains of erythrocyte membranes, thus producing the increase in 18:2ω6 and 20:4ω6 levels in the phospholipids of cell membranes. The diet supplemented with cholesterol causes an increase of 18:1ω9 and 20:3ω6 and decreases the amounts of 18:0 and 18:2ω6 in the erythrocyte phosphatidylcholine fraction in rats (37). In dogs, feeding an atherogenic diet produces an increase of 18:1ω9, 20:3ω9 and 20:3ω6 and a reduction of 18:2ω6 and 20:4ω6 in the lipids of mitochondria and microsomes of liver cells (38). In guinea pigs, the cholesterol supply causes a reduction of 16:0 and 18:0 and an increase of 18:1ω9, 20:3ω6 and 20:4ω6 in the lipids of erythrocytes (39). In rabbits, feeding cholesterol produces an increase of the cholesterol/phospholipid ratio (40).

The changes in lipid composition decrease above are accompanied by compensatory mechanisms tending to maintain the cell homeostasis. Brasitus et al. (36) showed that the increase in the amount of unsaturated fatty acids is compensated by the increase of cholesterol/phospholipid ratio. Schouten et al. (41) proved that an increase of 20:5ω3 is compensated by the decrease of 18:2ω6 in the phosphatidylcholine fraction of erythrocytes. Schouten et al. (41) stated that increase of the cholesterol/phospholipid ratio goes along with the compensatory decrease of 18:0 decrease of 18:0 and the increase of 18:2ω6. The increase of 20:3ω6 observed by the same team in other experiments (37,41) is an adaptive change in response to the increase of the cholesterol/phospholipid ratio. The increased cholesterol/phospholipid ratio in erythrocytes of the guinea pig fed an atherogenic diet produces the compensatory decrease of 16:0 and 18:0 and an increase of 20:3ω6 and 20:4ω6 (39). Similar changes were observed by Gibson et al. in lipids of subcellular fractions of liver, kidney, brain and heart in rats (42).

The stated decrease of 16:0 and 18:0 and the increase of 18:2ω6 and 20:3ω6 in the erythrocyte phospholipid fraction in atherosclerotic patients also can be regarded as compensatory changes aimed at the maintenance of adequate physico-chemical properties of the cell membrane in response to the increased cholesterol/phospholipid and sphingomyelin/phospholipidcholine ratios. Popp-Snijders et al. recently showed that the compensatory changes described above are sufficient for maintaining adequate fluidity of erythrocyte membrane (43).

The compensatory mechanisms that we have observed may explain previously stated (14) lack of differences in the strength of the cytoprotective effect of prostaglandins on erythrocytes of healthy persons and patients with atherosclerosis. The investigations of Borochov and Shinitzky (44) suggest that the availability of surface receptors of red blood cell membrane depends on its fluidity. The compensatory changes of the kind described above can be the reason for identical availability of receptors for prostaglandins in both cases.

REFERENCES

1. Thompson, N.L., and Axelrod, D. (1980) *Biochim. Biophys. Acta 597,* 155-165.
2. Hsia, J.C., and Boggs, J.M. (1972) *Biochim. Biophys. Acta 266,* 18-25.
3. McIntosh, T.J. (1978) *Biochim. Biophys. Acta 513,* 43-58.
4. Cooper, R.A. (1978) *J. Supramol. Struct. 8,* 413-430.
5. Wiley, J.S., and Cooper, R.A. (1975) *Biochim. Biophys. Acta 413,* 425-431.
6. Giraud, F., Claret, M., Bruckdorfer, K.R., and Chailley, B. (1981) *Biochim. Biophys. Acta 647,* 249-258.
7. Kroes, J., and Oswald, R. (1971) *Biochim. Biophys. Acta 249,* 647-650.
8. Papahadjopoulos, D., Nir, S., and Ohki, S. (1972) *Biochim. Biophys. Acta 266,* 561-583.
9. Grunze, M., Forst, B., and Deuticke, B. (1980) *Biochim. Biophys. Acta 600,* 860-869.
10. Cooper, R.A., Arner, E.C., Wiley, J.S., and Shattil, S.J. (1975) *J. Clin. Invest. 55,* 115-126.
11. Cooper, R.A., Diloy-Puray, M., Lando, P., and Greenberg, M.S. (1972) *J. Clin. Invest. 51,* 3182-3192.
12. Cooper, R.A. (1977) *N. Engl. J. Med. 297,* 371-377.
13. Gottlieb, M.H. (1980) *Biochim. Biophys. Acta 600,* 530-541.
14. Michalak, J., Kadziolka, A., Ledwozyw, A., and Madejczyk, A. (1986) *Pol. Tyg. Lek. 41,* 1585-1588.
15. Rapp, W., and Kahlke, W. (1968) *Clin. Chim Acta 19,* 493-498.
16. Fredrickson, D.S., Levy, R.I., and Lees, R.S. (1967) *Engl. J. Med. 276,* 34-44, 94-103, 148-156, 215-224, 273-281.
17. Szostak, W.B., Cybulska, B., Sekula, W. (1985) *Polish J. Human Nutr. Metab. 12,* 9-15.
18. Bouhours, J.F., and Bouhours, D. (1984) *J. Lipid Res. 25,* 613-619.
19. Stein, J., and Smith, G. (1982) *Techn. Lipid Membr. Biochem. B403,* 1-15.
20. Zlatkis, A., Zak, B., and Boyle, A.J. (1953) *J. Lab. Clin. Med. 41,* 486-492.
21. Bartlett, G.R. (1959) *J. Biol. Chem. 234,* 466-468.
22. Allen, K.G., MacGee, J., Fellows, C.E., Tornheim, P.A., and Wagner, K.R. (1984) *J. Chromatogr. 309,* 33-42.
23. Morrison, W.R., and Smith, L.H. (1964) *J. Lipid Res. 5,* 600-608.
24. Ledwozyw, A., Pruszkowska, R., and Kadziolka, A. (1984) *Acta Physiol. Pol. 35,* 403-411.
25. Oktaba, W. (1976) in *Mathematical Statistics,* 4th edn., pp. 115-137, PWN, Warszawa, Poland.
26. Cooper, R.A., Durocher, J.R., and Leslie, M.H. (1977) *J. Clin. Invest. 60,* 115-121.
27. Cooper, R.A. (1969) *J. Clin. Invest. 48,* 1820-1831.
28. Cooper, R.A., Kimball, D.B., and Durocher, J.R. (1974) *N. Engl. J. Med. 290,* 1279-1284.
29. Chobanian, A.V., and Hollander, W. (1966) *J. Clin. Invest. 45,* 932-938.
30. Böttcher, C.J., and Van Gent, C.M. (1961) *J. Atheroscler. Res. 1,* 36-46.
31. Cullis, P.R., and Hope, M.J. (1980) *Biochim. Biophys. Acta 597,* 533-542.
32. Ehrström, M., Harms-Ringdahl, M., and Allig, C. (1981) *Biochim. Biophys. Acta 644,* 175-182.

33. Cullis, P.R., and De Kruijff, B. (1978) *Biochim. Biophys. Acta 507,* 207-218.
34. Live, A., Kuiper, P.J.C., and Meyerstein, N. (1972) *Biochim. Biophys. Acta 255,* 744-750.
35. Schrade, W., Biegler, R., and Böhle, E. (1961) *J. Atheroscler. Res. 1,* 47-61.
36. Brasitus, T.A., Davidson, N.O., and Schachter, D. (1985) *Biochim. Biophys. Acta 812,* 460-472.
37. Popp-Snijders, C., Schouten, J.A., De Jong, A.P., and Van der Veen, E.A. (1984) *Scand. J. Clin. Lab. Invest. 44,* 39-46.
38. Schouten, J.A., Beynen, A.C., Popp-Snijders, C., and Mulder, C. (1985) *Nutr. Rep. Int. 31,* 229-236.
39. Balachandran, R., and Ehrhart, L.A. (1975) *Proc. Soc. Exp. Biol. Med. 149,* 29-34.
40. Oswald, R., and Shannon, A. (1964) *Biochem. J. 91,* 146-154.
41. Schouten, J.A., Beynen, A.C., Popp-Snijders, C., and Van der Veen, E.A. (1984) *Nutr. Rep. Int. 29,* 1223-1232.
42. Gibson, R.A., McMurchie, E.J., Charnock, J.S., and Kneebone, G.M. (1984) *Lipids 19,* 942-951.
43. Popp-Snijders, C., Schouten, J.A., van Blitterswijk, W.J., and Van der Veen, E.A. (1986) *Biochim. Biophys. Acta 854,* 31-37.
44. Borochov, H., and Shinitzky, M. (1976) *Proc. Natl. Acad. Sci. USA 73,* 4526-4530.

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METHODS

Composition of Mixed Octadecadienoates via Ozonolysis, Chromatography and Computer Solution of Linear Equations[1]

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An approach to the analysis of 55 possible nonconjugated positional isomers of octadecadienoic acid is described and tested with mixtures of individual synthetic methyl esters. In the first example, by ozonolysis a seven-component mixture consisting of *cis,cis* 5,12-, 6,10-, 6,11-, 6,12-, 7,12-, 8,12-, and 9,12-octadecadienoates was converted to aldehydes, aldehyde-esters and dialdehydes. These fragments were separated on a 50 m × 0.2 mm free fatty acid phase (FFAP) vitreous silica capillary column. Equations for an arbitrarily restricted 12 × 15 matrix of linear simultaneous equations and a computer solution of the matrix provided the composition of the initial methyl octadecadienoate mixture. The power and significance of this method became apparent with the observation that only two of the seven isomers in the known mixture were resolved as single peaks by state-of-the-art capillary gas chromatography, but all seven were identified and estimated with acceptable error by the ozonolysis-capillary gas chromatography-computer procedure. In a generalized approach to the analysis of the 55 possible nonconjugated isomers, a computer program selects the appropriate matrix of linear simultaneous equations based on the aldehyde data supplied by the analyst. Twenty of 21 combinations of seven isomeric esters taken five at a time have been analyzed to assess the efficiency of the method. To illustrate applicability at this stage of development, the method has been used to analyze the diene products of the hydrazine reduction of γ-linolenic acid and the diene products from the biological desaturation of isomeric monoenes. The possibility of distinguishing geometric and positional isomers of 18:2 has been opened by the observed separation of *cis*- and *trans*-unsaturated aldehydes and aldehyde-esters.

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Methods for the analysis of positional isomers of octadecadienoates as they occur in mixtures of chemical or biological origin generally are lacking (1). A procedure for the analysis of 18:2 isomers from partially hydrogenated fats has been described (2) but was not applicable to samples of biological origin because the range of double-bond positions permitted was too limited. It involved ozonization and reduction to form alcohols, gas chromatographic separation of the alcohols, alcohol-esters, dialcohol fragments and, finally, mathematically identifying the component 18:2 isomers in the original mixture and estimating their proportions from the proportions of the fragments produced.

In this report, an improved procedure is proposed for the analysis of a wider range of isomers such as may be expected in biological studies (3). Based on the capillary gas chromatographic (CGC) separation and measurement of the aldehydes, which are more quantitatively formed from the ozonides than are alcohols, the method permits analysis of mixtures of isomeric nonconjugated dienoic acids with double bonds ranging from the Δ3 to Δ15 positions, limits that are reasonable for biological samples. The method has been tested previously (4) with seven known mixtures of synthetic isomeric dienoic fatty acid methyl esters (5) and upon the diene products of the hydrazine reduction of γ-linolenic acid for which the products are predictable, and those preliminary results appeared in the Congress' Proceedings (4). This final report is based mainly upon new data in which alkaline isomerization was used for measurement of 1,4-dienes rather than malondialdehyde and on tests of the method with 21 known model mixtures of 18:2 acids.

EXPERIMENTAL

Synthetic mixtures. Synthetic isomers of *cis,cis*-18:2 (5) were used to prepare mixtures of known composition for analysis and development of procedure. The initial mixture consisted of equal amounts by weight of *cis,cis*-5,12-, 6,10-, 6,11-, 6,12-, 7,12-, 8,12- and 9,12-octadecadienoic acid methyl esters. The major test of the approach was by a series of 21 synthetic equimixtures of the same seven isomeric esters taken five at a time.

Ozonizations. Ozonizations were performed by bubbling a stream of 10% O_3 in oxygen (50 ml/min) through petroleum ether solutions (ca. 20–100 μg of esters in 20–100 μl) cooled by a CO_2-acetone bath. Ozone was produced in a small glow discharge generator powered by a Tesla coil (6) or in a Bonner-type generator (7). After partial evaporation of the petroleum ether with a stream of N_2 at room temperature, an amount of triphenylphosphine in diethyl ether (8) twice the sample weight was added to reduce the ozonides to aldehydic products.

CGC separations. CGC separations of individual aldehyde (A), aldehyde-ester (AE) and dialdehyde (AA) fragments were performed using a 50 m × 0.2 mm free fatty acid phase (FFAP) (Carbowax) column (Supelco, Inc., Bellefonte, PA) and a 50 m × 0.2 mm SP 2330 vitreous silica column (Scientific Glass Engineering, Inc., Austin, TX). The FFAP capillary column was used isothermally at 185 C and 190 C to partially separate the

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Abbreviations: CGC, capillary gas chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; A, aldehyde; AA, dialdehyde; AE, aldehyde ester; FFAP, free fatty acid phase; PHSO, partially hydrogenated soybean oil; PL, phospholipids.

initial mixtures of methyl octadecadienoates (Table 1). To separate aldehydic products, both the SP 2330 and FFAP columns were programmed from 40 C to 220 C at a rate of 6 C/min in a Packard Model 428 gas chromatograph equipped with a flame ionization detector. Peak areas and retention times were measured by an IMSAI 8080 microprocessor.

Conjugatable double bonds. The percentage of methylene-interrupted dienes was determined as a preferred alternative to or in addition to the CGC measurement of malondialdehyde (AA3), which is poorly detected because it has only one ionizable carbon atom. The alkaline conjugation was performed in the injection port with tetramethylammonium hydroxide, and the nonconjugated dienes were separated and measured by CGC (9). The malondialdehyde equivalent to the conjugatable dienes was calculated and used in the matrix solution.

Mathematical procedures for the seven-component initial equimixture. The analytical problem involves a matrix of linear simultaneous equations relating the occurrence and proportions of the several possible A, AA and AE fragments arising from a broad range of isomeric 18:2 structures (2). This matrix is shown in Figure 1. Because conjugated isomers rarely are found in animal lipids,

TABLE 1

Comparison of Analytical Methods of the Seven-component Synthetic Mixture of Dienes

	Weight (%)	CGC (Fig. 2) (%)	Ozonolysis direct calculation (%)	Ozonolysis restricted matrix solution[a] (%)	Ozonolysis unrestricted matrix solution[b] (%)
5,10-				3.3	2.1 ± 3.1
5,11-					3.9 ± 3.0
5,12-	14.3	46.3[c]	23.2, 13.2	16.6	15.2 ± .05
6,10-	14.3		15.9	13.4	15.0 ± 3.0
6,11-	14.3	10.3	16.8	13.9	11.1 ± 0.0
6,12-	14.3	26.4[d]	15.1	13.4	12.0 ± 3.0
7,10-				1.5	
7,11-					1.9 ± 3.0
7,12-	14.3		13.1	9.9	11.7 ± 3.0
8,11-					
8,12-	14.3		12.6	15.9	14.2 ± .05
9,12-	14.3	16.0	12.1, 13.6	12.1	12.9 ± 0.0

[a]Restricted to isomers permitted by the single-skip hypothesis.

[b]Average ± S.D. of six solutions of minimum error considering all 66 combinations of 12 isomers taken 10 at a time.

[c]Includes 6,10-, 5,12-, and 7,12-.

[d]Includes 6,12- and 8,12-.

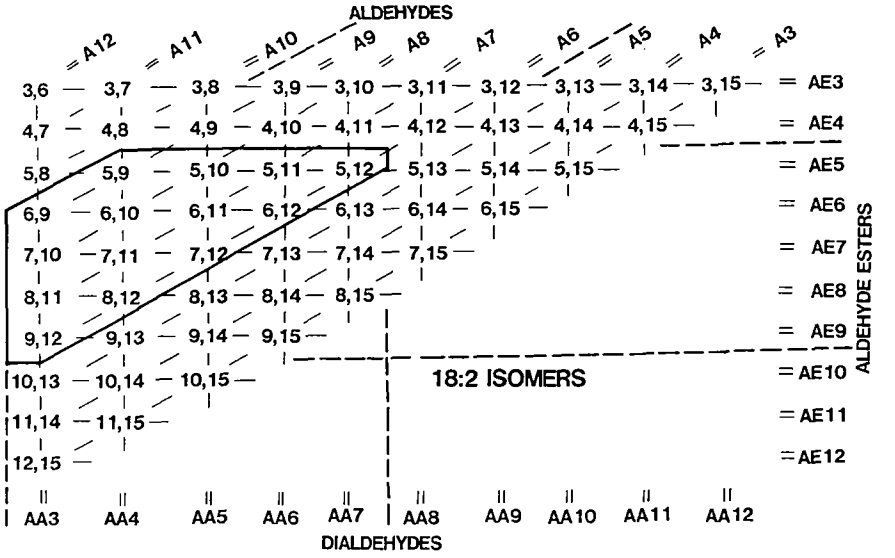

FIG. 1. Triangular representation of 30 linear simultaneous equations relating 55 nonconjugated 18:2 isomers to A, AA and AE fragments. AE homologs are summations horizontally; AA homologs are vertical summations; A homologs are diagonal summations, as shown. Enclosed area includes the equations limited by the range of analytical data supplied.

these isomers were excluded from consideration. There are 55 positional, nonconjugated isomers of 18:2 possible, with double bonds ranging from positions Δ3 to Δ15. Isomers of 18:2 outside this range also are very unlikely in natural samples, and even those 18:1 isomers from which they might be derived occur in hydrogenated fats in vanishingly small proportions. For these 55 isomers, there are at a maximum only 30 aldehydic products possible by ozonolysis and reduction. Obviously, such a matrix cannot be solved nor need it be in most practical samples because relatively few isomers occur in any given system. In the earlier application to the diene structures expected in partially hydrogenated oils (2), the "single skip principle" was applied, in which only one of the two double bonds of a methylene-interrupted diene is expected to shift in a given molecule during hydrogenation. Application of the single skip principle here reduced the matrix to 15 equations, relating 12 unknowns that should permit mathematical solutions. Applying a narrower matrix for the range Δ5 to Δ12, covering the range of double bond positions occurring in the model esters, the aldehydic products of an equimixture of the seven synthetic dienes were analyzed (Fig. 2), the products identified and the matrix equations solved as shown in Figure 3.

The restriction of the single skip that applies to hydrogenation may not be valid for unknown biological systems, and a more generalized procedure for limiting the numbers of equations was needed. Accordingly, an initial subroutine, BRAIN, to the computer program was devised to select the appropriate equations involving only those isomers that could yield the aldehydic products observed. This same elimination of unneeded equations can also be performed graphically, as shown in Figure 1 by the broken lines that intersect to include an area (solid lines) of 14 unknowns and 14 equations required by AE5 to AE9, AA3 to AA7 and A6 to A9. This reduction in matrix size is followed by a routine, DIENES, which makes further deletions of isomers to be considered by deletion of the minor aldehydic components, if necessary, to achieve a solution. It also employs the subroutine, ORTHO (2), which solves the remaining matrix iteratively. This composite program is available from the first author. A first test of this generalized approach was made with the same equimixture of seven available synthetic isomers of 18:2 whose double bonds occurred at carbons 5 through 12. Despite the 13 equations relating 12 unknowns, this matrix was not solved directly for the seven-component mixture because of redundancies (2). Therefore, a strategy successively considering only 10 of the 12 isomers at a time and evaluating the results for minimum error was undertaken. The average for six minimum error solutions is given in the last column of Table 1. This strategy, while acceptable for knowns, is not justifiable for unknowns.

Mathematical procedure for the 21 five-component mixtures of seven esters. To evaluate accuracy and precision of the general analytical method and matrix system, a series of 21 mixtures of the seven esters taken five at a time was made as indicated in Table 2. Computer

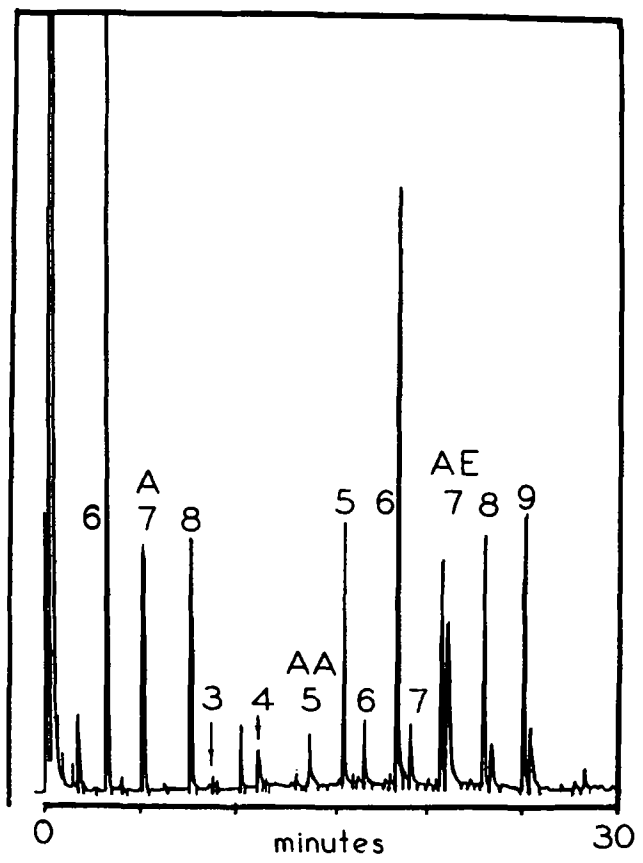

FIG. 2. Temperature-programmed capillary gas chromatogram of the aldehydic products from the ozonization of the seven-component synthetic mixture of diene isomers using a 0.2 mm × 50 m FFAP vitreous silica column. AE, aldehyde esters; AA, dialdehydes; A, aldehydes all with numbers indicating chain lengths. (50 M FFAP column programmed 40 C to 220 C at 6 C/min.)

A

INPUT DATA DATA AND EQUATIONS

	05	8	5	9	510	512	6	9	610	611	612	710	712	812	912	
23.200	1.	1.	1.	1.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.AE5
39.000	0.	0.	0.	0.	1.	1.	1.	1.	1.	0.	0.	0.	0.	0.	0.	0.AE6
13.100	0.	0.	0.	0.	0.	0.	0.	0.	1.	1.	0.	0.	0.	0.	0.	0.AE7
12.600	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	1.	0.	0.	0.	0.	0.AE8
12.100	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	1.	0.	0.	0.	0.AE9
13.600	1.	0.	0.	0.	1.	0.	0.	0.	1.	0.	0.	1.	0.	0.	0.	0.AA3
32.700	0.	1.	0.	0.	0.	1.	0.	0.	0.	0.	1.	0.	0.	0.	0.	0.AA4
25.400	0.	0.	1.	0.	0.	0.	1.	0.	0.	1.	0.	0.	0.	0.	0.	0.AA5
15.100	0.	0.	0.	0.	0.	0.	0.	1.	0.	0.	0.	0.	0.	0.	0.	0.AA6
13.200	0.	0.	0.	1.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.AA7
67.200	0.	0.	0.	1.	0.	0.	0.	1.	0.	1.	1.	1.	1.	0.	0.	0.A6
16.800	0.	0.	0.	0.	0.	0.	1.	0.	0.	0.	0.	0.	0.	0.	0.	0.A7
15.900	0.	0.	1.	0.	0.	1.	0.	0.	1.	0.	0.	0.	0.	0.	0.	0.A8
.000	0.	1.	0.	0.	1.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.A9
.000	1.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.A10
100.000	1.	1.	1.	1.	1.	1.	1.	1.	1.	1.	1.	1.	1.	0.		TOTAL

B

COMPD	CALC PERCENT	NORM PERCENT
5, 8	1.342	1.320
5, 9	6.845	6.736
5,10	-.180	-.177
5,12	14.522	14.289
6, 9	-6.002	-5.906
6,10	12.258	12.062
6,11	16.647	16.381
6,12	15.924	15.669
7,10	4.167	4.101
7,12	9.258	9.110
8,12	13.424	13.209
9,12	13.422	13.207
TOTAL	101.627	100.000

C

COMPD	CALC PERCENT	NORM PERCENT
5, 8	1.409	1.394
5, 9	3.577	3.541
5,12	15.912	15.751
6,10	13.562	13.424
6,11	14.221	14.077
6,12	13.364	13.228
7,10	1.467	1.452
7,12	11.611	11.493
8,12	14.286	14.141
9,12	11.617	11.499
TOTAL	101.026	100.000

FIG. 3. Computer solution of matrix for the seven-component synthetic mixture. A, input data and linear equations in determinant format; a one or zero in the body of the table indicates presence or absence, respectively, of the isomer indicated at the top of the column in the linear equation of the row; B, first approximation; C, results of final iteration.

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TABLE 2

Summary of Data from 21 Model Mixtures of Five Components of Isomeric Octadecadienoates

	Gravimetric composition of model mixture isomers													Calculated analytical composition of model mixture isomers										
Samples	5,10	5,11	A 5,12	B 6,10	C 6,11	D 6,12	7,10	7,11	E 7,12	8,11	F 8,12	G 9,12	Samples	5,10	5,11	A 5,12	B 6,10	C 6,11	D 6,12	7,10	7,11	E 7,12	8,11	F 8,12	G 9,12
-AC			—	20.0	—	20.0			20.0		20.0	20.0	-AC	—	—	—	17.7	—	21.5	6.7	—	23.8	—	14.6	15.7
-AE			—	20.0	20.0	20.0			—		20.0	20.0	-AE	—	—	—	15.3	26.2	13.7	—	—	—	—	16.9	27.9
-AG			—	20.0	20.0	20.0			20.0		20.0	—	-AG	—	—	—	22.0	10.6	25.2	—	8.9	23.3	—	10.0	—
-BD			20.0	—	20.0	—			20.0		20.0	20.0	-BD	—	—	20.0	—	16.2	—	—	6.0	29.8	1.1	13.4	13.6
													-BD	—	—	30.9	—	14.0	—	—	10.0	13.6	—	14.5	17.0
-BF			20.0	—	20.0	20.0			—		20.0	20.0	-BF	—	5.0	19.4	—	11.4	15.9	—	—	21.6	—	—	23.8
													-BF	—	4.4	24.6	—	12.2	19.3	—	—	25.0	—	—	14.6
-CE			20.0	20.0	—	20.0			—		20.0	20.0	-CE	—	—	17.1	21.1	—	24.8	—	—	—	—	12.4	24.6
													-CE	—	—	17.6	21.3	—	25.7	—	—	—	—	13.2	22.1
													-CE	—	4.0	22.1	18.7	—	21.2	—	—	—	8.0	9.4	16.6
													-CE	—	—	24.1	19.9	—	24.0	—	—	—	—	12.0	20.5
													-CE	—	—	11.9	15.2	—	26.1	—	—	—	—	14.2	23.4
-CG			20.0	20.0	—	20.0			20.0		20.0	—	-CG	2.3	—	21.1	9.0	—	24.8	16.5	—	9.6	—	11.7	—
													-CG	—	—	23.2	23.9	—	27.7	—	—	18.3	—	11.0	—
-DF			20.0	20.0	20.0	—			20.0		—	20.0	-DF	—	—	27.4	17.1	17.4	—	5.6	—	17.2	—	—	14.6
													-DF	—	—	11.9	24.1	10.1	—	—	9.2	22.5	—	—	17.3
-EF			20.0	20.0	20.0	20.0			—		—	20.0	-EF	1.2	—	14.5	21.3	17.9	20.0	—	—	—	—	—	20.2
-FG			20.0	20.0	20.0	20.0			20.0		—	—	-FG	—	—	—	—	—	—	—	—	—	—	—	—
-AB			—	—	20.0	20.0			20.0		20.0	20.0	-AB	—	—	—	—	20.4	15.2	—	—	23.0	3.3	20.2	18.0
-AD			—	20.0	20.0	—			20.0		20.0	20.0	-AD	—	—	—	16.6	18.5	—	—	5.8	13.6	—	22.6	22.8
-AF			—	20.0	20.0	20.0			20.0		—	20.0	-AF	—	—	—	21.2	23.3	14.3	—	—	16.7	—	—	24.5
-BC			20.0	—	—	20.0			20.0		20.0	20.0	-BC	—	—	23.0	—	—	20.5	—	—	23.1	—	16.6	16.8
-BE			20.0	—	20.0	20.0			—		20.0	20.0	-BE	—	—	16.8	—	26.7	10.3	—	—	—	—	26.8	19.0
-BG			20.0	—	20.0	20.0			20.0		20.0	—	-BG	—	—	19.0	—	20.3	18.9	—	0.1	28.4	—	13.4	—
-CD			20.0	20.0	—	—			20.0		20.0	20.0	-CD	3.8	—	15.2	16.7	—	—	0.3	—	29.5	—	13.7	20.8
-CF			20.0	20.0	—	20.0			20.0		—	20.0	-CF	—	—	18.1	17.4	—	14.2	2.9	—	26.6	—	—	20.6
-DE			20.0	20.0	20.0	—			—		20.0	20.0	-DE	0.5	—	18.0	23.9	12.6	—	—	—	—	3.6	21.5	19.4
-DG			20.0	20.0	20.0	—			20.0		20.0	—	-DG	—	—	19.6	20.5	15.1	—	—	4.9	22.9	—	16.9	—
-EG			20.0	20.0	20.0	20.0			—		20.0		-EG	6.7	—	16.6	11.4	22.6	19.1	—	—	—	—	23.6	—

solutions of 20 of these 21 mixtures were successful using analytical input data from the model mixtures. The combination omitting esters F (8,12-) and G (9,12-), designated in Table 2 as -FG, was not solved by manual algebra or by our direct computer methods. The combination -BC is of particular interest because the solution was obvious by direct inspection of either the AA or the AE data, uniquely identifying esters present.

In the first test of the entire procedure, nine dispersed combinations of five isomers listed in Table 2 were chosen for analysis. CGC analyses of the methyl esters were made first to see if any of the combinations could be analyzed without the ozonization-computer matrix procedure. None of the mixtures was separable into its five components. After ozonization, reduction and CGC of the aldehydic products, computer solutions of the matrices were calculated, and the standard deviations of the values for each isomer occurring in the many combinations were calculated. In these calculations, percentage of conjugatable isomers by alkali isomerization-CGC (9) was used in lieu of measurement of percent malondialdehyde by ozonolysis-CGC.

Following the discovery that the -FG combination could not be solved directly, the remaining 11 combinations of the 21 were analyzed by the ozonization-computer procedure. Spreadsheets were exploited for the laborious calculation of corrected mole percentage and integrated graphics for stacked-bar histograms were used as helps in development. The data from this extended series of model mixtures also are in Table 2.

Solutions of linear simultaneous equations were performed using the improved FORTRAN program, which eliminated from consideration those isomers for which no experimental data existed and deleted the related equations from the matrix. The program minimized the sum-squared error of all possible solutions by an iterative process, and printed out the observed input data, the values for the data used, and the absolute error and the percentage error between the latter two. When a negative value was found, the program set that isomer at zero, and the calculation was repeated. This program, the spreadsheets and the graphics were run on an Apple IIe computer (10). The program is available from the Hormel Institute[2] (10).

Standard dienes. The synthetic isomeric 18:2 fatty acid methyl esters (5) had *cis-cis* double bonds at the 5,12-, 6,10-, 6,11-, 6,12-, 7,12- and 8,12- positions. In addition, methyl linoleate (9,12-18:2) and methyl linoelaidate (t9,t12-) were obtained from NuChek Prep., Inc. (Elysian, MN). These isomers covered the range of double bond positions from Δ5 to Δ12.

Hydrazine-reduced γ-linolenic acid. Dienes from hydrazine-reduced γ-linolenic acid were prepared in the following way: to 10 μl of 6,9,12-octadecatrienoic acid (γ-linolenic acid, 18:3ω6) dissolved in 1.6 ml of methyl alcohol, 0.3 ml of hydrazine hydrate (64%) was added; and oxygen gas (50 ml/min) then was bubbled for 10 min into the reaction mixture in a 20 C water bath. The reaction mixture was then acidified with 3 N methanolic HCl, extracted with petroleum ether, washed with water, dried, esterified with BF_3-methanol (14% w/v), extracted with petroleum ether and washed. The resultant methyl esters were separated by thin layer chromatography (TLC) on 2% silver nitrate-treated Silica Gel H plates using petroleum ether-diethyl ether (100:10, v/v) as solvent to obtain the diene fraction, which theoretically should be equal parts of 6,9-, 9,12- and 6,12-18:2.

Liver phospholipids (PL). Liver PL from a rat fed 18% partially hydrogenated soybean oil (PHSO) were isolated and used to test the analytical method on a biological sample likely to contain 18:2 isomers (11). After conversion to methyl esters, the diene fraction was isolated by $AgNO_3$-TLC, and the C_{18} dienes were separated from C_{20} dienes by high performance liquid chromatography (HPLC) on an ALTEX C-18 column using 95% methanol.

RESULTS

Analysis of the seven-component known mixture. CGC of an equimixture of the seven isomeric methyl octadecadienoates listed above showed three major unresolved peaks and a fourth peak that was more completely resolved and integrated (Fig. 4). From the retention times for the individual components (4), the identifications of components within each peak could be made.

The equimixture of the seven isomeric methyl octadecadienoates also was ozonized, reduced, and the resultant AE, AA and A fragments were separated by temperature-programmed CGC (Fig. 2). Identifications of aldehydic fragments were made by ozonizing the individual isomers and CGC separation of the resulting aldehydic fragments. Plots of elutin temperature vs chain length of A, AE and AA fragments each gave a linear relationship (4,12). This

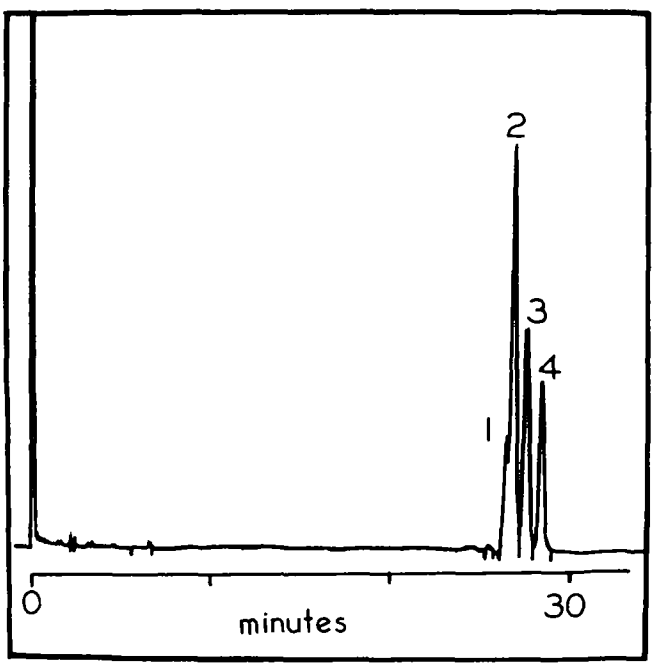

FIG. 4. CGC separation of the seven-component synthetic mixture of methyl octadecadienoates. Peak 1: 6,11-isomer. Peak 2: 6,10-, 5,12- and 7,12-isomers. Peak 3: 6,12- and 8,12-isomers. Peak 4: 9,12-isomer. (50 M FFAP column at 190 C.)

[2]All necessary FORTRAN programming and instructions for use on Apple II computers and a demonstration routine on disk will be supplied by the first author upon request. (Include a blank 5¼" diskette and a self-addressed, stamped mailing carton.)

includes the peak corresponding to malondialdehyde, which is small because of its one ionizable carbon atom, and which therefore is less reliable for quantitative purposes. It has been used previously to qualitatively establish the structure of methylene-interrupted double bonds in individual trienes and tetraenes (13). Only those peaks that fell where expected were considered in the calculations, and spurious peaks were ignored. Of particular concern was the large unidentified peak that sometimes followed AE7. Our study of the kinetics of ozonization indicated that it forms in proportion to the AE fragments. In packed 1/8″ columns, this peak is not resolved from AE7 and it gives such large and erroneous values for AE7 to make the data arising from packed columns invalid, and to make CGC the tool of necessity.

Table 3 gives the uncorrected areas from Figure 2, the areas corrected for ionizable carbon atoms (14), the molar percentages adding up to 100% for each type of fragment and, finally, the identities of the isomers contributing to each peak. These molar percentage values were the input data used in the computer program. The percentage of conjugatable diene obtained by injection port alkali isomerization-CGC was 13.7% corresponding to 13.6% in Table 3 for AA3 (9).

A computer printout showing these input data (first column) and the matrix of 18:2 isomers derived from them are shown in Figure 3A. The first solution (Fig. 3B) gave two negative values that were eliminated in the next iteration. Figure 3C shows the final solution.

In the equimixture analyzed, each isomer has as its unique expression, one AE or one AA or both (Table 1). Therefore, a qualitative analysis should be feasible from the AE and A produced, and a quantitative analysis is feasible from the proportions of the products (Table 1). However, in those instances in which an isomer could be measured independently by two different aldehydic fragments, discrepancies between the two calculations were apparent (Table 3). For example, measurement of 5,12-18:2 by the AE5 alone gave 23.2%, but using the AA7 alone gave 13.2%, an example of the experimental error of analysis by direct calculation. Table 1 compares the composition of the known mixture of standard 18:2 isomers by gravimetry with its analysis by the methods of CGC, by direct calculation, by computer matrix solution with a restricted matrix, and in the last column, by an unrestricted matrix that examined all 66 possible combinations of 12 esters taken 10 at a time, and selected six solutions of minimum error for averaging.

CGC analysis of the 21 five-component known mixtures of the seven esters. Direct CGC of the 10 experimental mixtures -AC, -AE, -AG, -BD, -BE, -CE, -CG, -DF, -EF and -FG resolved only two peaks in four of the mixtures, three peaks in five, and four peaks in one. Four peaks also were found in the first seven-component mixture described above, but only the 9,12 isomer was clearly separated in all mixtures. These measurements showed the inadequacy of current CGC for separation of 18:2 isomers.

Ozonolysis, reduction, CGC of aldehydes and computer matrix solutions of the data gave for the first nine equimixtures an average value of 19.3% ± 5.1% (S.D., n = 9). The difference between this average and the expected 20% for each is accounted for by small percentages of isomers that were required by the computer solution for minimizing the error of the input data. In these calculations, the percentage of conjugatable dienes was used in lieu of the percentage of malondialdehyde by CGC measurement. The alkali conjugation-CGC analyses on eight mixtures that contained linoleate (20%) had an average percentage value of 17.6% ± 2.4 (S.D.) conjugatable

TABLE 3

Aldehyde Fragments Obtained from Ozonolysis and Reduction of the Seven-component 18:2 Mixture

Fragments	Temp.	Time	Peak area	Ionized carbons	Corrected area	Mol %	Contributing diene(s)
AE9	203.2	25.53	7,101	8	888	12.1	9,12-
AE8	191.4	23.57	6,454	7	922	12.6	8,12-
AE7	179.1	21.52	5,786	6	964	13.1	7,12-
AE6	166.4	19.40	114,320	5	2,864	39.0	6,10-, 6,11-, 6,12-
AE5	150.9	16.81	6,821	4	1,705	23.2	5,12-
					7,343	100.0	
AA7	170.2	20.04	1,541	5	308	13.2	5,12-
AA6	157.0	17.83	1,411	4	353	15.1	6,12-
AA5	141.0	15.16	1,786	3	595	25.4	6,11-, 7,12-
AA4	126.4	12.73	1,529	2	764	32.7	6,10-, 8,12-
AA3	113.2	10.54	318	1	318	13.6	9,12-
					2,338	100.0	
A6	82.1	5.35	18,413	5	3,683	67.2	5,12-, 6,12-, 7,12-, 8,12-, 9,12-
A7	93.4	7.23	5,537	6	922	16.8	6,11-
A8	107.1	9.52	6,099	7	871	15.9	6,10-
					5,476	99.9	

isomers. It should be noted that with the highly efficient inlet splitters of modern CGCs and their inherently low residence time, the conversion of linoleate to conjugated diene was only 64% compared with 95–96% for direct injection in the old-style packed column GCs and/or the micro reactor apparatus as originally published (9). The 64% correction factor found for the conjugation of methyl linoleate brought conjugated diene values for these known mixtures near theory. In the analyses of the remaining 11 combinations, theoretical values of 20% for malondialdehyde were used.

As noted above, it was found that neither the computer nor manual algebraic attempts could solve one of the combinations (-FG); therefore, it became mandatory to run the remaining 11 combinations (-AB, -AD, -AF, -BC, -BE, -BG, -CD, -CF, -DE, -DG and -EG) through the matrix procedure. Spreadsheets were used for routine calculations and conversions of CGC areas to percentage mole fractions.

Much of the assignable error of the final calculation arose from error in input data. This became clear when stacked-bar histograms were constructed (directly from the spreadsheet). The heights of bars representing amounts of individual AE, AA and A analyses should be 0%, 20% or multiples of 20% up to 100%. Variability in input data from the 20% multiples was apparent and inevitably contributed to the error of computer output data.

Partial ozonization of a methyl linoleate-methyl linolelaidate mixture. Partial ozonization of *cis*- and *trans*-isomers of 18:2 yields mixtures of *cis*- and *trans*-unsaturated aldehydes and aldehyde esters that are distinguishable by CGC. An equimixture of these isomers was ozonized, leaving 3.1 and 3.9 mol % of unreacted methyl linolelaidate and methyl linoleate, respectively (12). CGC (SP 2330 temperature-programmed from 40–240 C at a rate of 2 C/min) gave two peaks in the temperature/time window expected for unsaturated aldehydes of 12 carbon atom length—the first, 6.2 mol % *trans*-3-dodecenal, the second 2.5 mol % of *cis*-3-dodecenal. Peaks of 1.4 and 1.1 mol % were present in the region expected for 3-*trans* C_{12} aldester and 3-*cis* C_{12} aldester just preceding the elution of the two parent ester peaks. From this and other partial ozonizations of the linoleate-linolelaidate mixture, it is apparent that *cis* and *trans* aldehydes and *cis* and *trans* aldehyde esters can be separated as well as the parent linoleate and linolelaidate on a CGC SP 2330 column.

Analysis of dienes from hydrazine-reduced γ-linolenic acid. The capillary chromatogram of the hydrazine reduction mixture is shown in Figure 5. All three expected isomers were detected, 6,9-, 6,12- and 9,12-18:2. Table 4 gives the compositions of the hydrazine reaction mixture before and after isolation of the dienes by $AgNO_3$-TLC. It compares the theoretical composition with analyses by direct CGC of the 18:2 isomers and by the ozonolysis-matrix procedure. CGC and ozonolysis matrix solution gave values that deviate similarly from theory.

Analysis of dienes from rat liver phospholipid. The capillary gas chromatogram for the 18:2 isomers isolated from liver PL of one rat fed PHSO showed three major peaks whose shapes indicated multiple unseparated isomers, which accounted for 25.3, 23.4 and 51.3% in order of their elution. After ozonization of the mixture, the following aldehydes were found: AE5, AE6, AE7, AE8, AE9, AA3, AA4, AA5, AA6, AA7, A6, A7, A8 and A9. The analysis of the 18:2 isomers by ozonolysis and matrix solution revealed at least five components, with values for 9,12-18:2 varying from 38.8 to 51.5% on replicates, to be compared with analysis by CGC (51.3%) and by total conjugatable dienes (53.4%). Application of the ozonolysis-matrix solution procedure to relevant biological problems will be the subject of a later report.

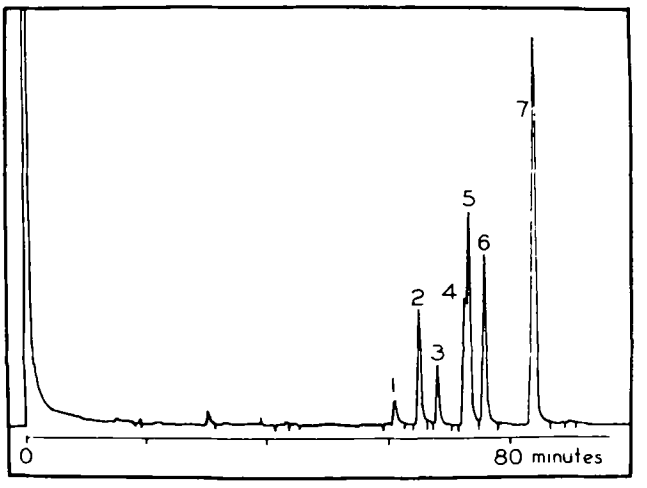

FIG. 5. CGC separation of the methyl esters from hydrazine reduction of γ-linolenic acid. Peak 1: 6-octadecenoate. Peak 2: 9-octadecenoate. Peak 3: 12-octadecenoate. Peak 4: 6,9-octadecadienoate. Peak 5: 6,12-octadecadienoate. Peak 6: 9,12-octadecadienoate. Peak 7: 6,9,12-octadecatrienoate. (50 M FFAP column, isothermal at 190 C.)

TABLE 4

Analyses of Isomeric 18:2 Products from Hydrazine Reduced γ-Linolenic Acid by CGC and by Ozonolysis-Matrix Solutions

Isomer	Total reaction mixture direct CGC[a]	Simple theory	CGC	Dienes by TLC ozonolysis-matrix solution
Monoenes				
6-	1.7			
9-	8.6			
12-	4.6			
Dienes				
6,9-	28.0 (10.8)[b]	33.3	25.0	22.2
6,12-	28.0 (17.2)[b]	33.3	33.3	32.0
9,12-	14.3	33.3	41.7	43.8
Triene				
6,9,12-	42.8			

[a]Figure 11.
[b]Estimated from peak heights.

DISCUSSION

The thesis that "Every Advance in Scientific Knowledge is First an Advance in Technique" has as its converse the reality that advances in scientific knowledge frequently must await developments in technique. Thus, study of the metabolism of isomers of linoleic acid is impeded by the absence of an adequate methodology for the analysis

of 18:2 isomers (1). It is this vacuum that the present preliminary studies attempt to fill so that future advances in scientific knowledge may be facilitated.

The only previous attempt at analysis of naturally occurring 18:2 isomers known to us attempted to match AE and A fragments formed from the mixtures, assuming methylene interruption (15). Without measurements of the AA fragments, such data can lead to ambiguous compositions of mixtures. The present method measures the dialdehyde fragments as well, and the malondialdehyde can be estimated from measurement of methylene-interrupted dienes by alkaline isomerization. The computer-assisted assembly of diene structures matches all three types of aldehyde fragments (A, AA, AE) derived from a dienoic acid. Our analysis of the known seven-component mixture showed an average error of ±1.2% on true gravimentric values of 14.3% or a relative error of 8.4% (Table 1). For 28 analyses of the 20 combinations of five pure esters (Table 2), an average percentage content of the individual components (present in all samples at 20% each) was estimated to be 19.0% ± 0.43 (S.D., n = 140), or a maximum relative error of about 25%. The average of components detected and reported but not actually present in the samples was 0.57% ± 0.14 (S.E.M., n = 140). Thus, the chance of missing a major component or of reporting an absent component is very small. The computer deletion of components less than 2% of the total to achieve convergence prevented gross errors of this kind. In the analysis of the products of hydrazine reduction of γ-linolenic acid, the matrix solution gave an average error of ±1.57% on an average theoretical value of 33.3%, or a relative error of 4.7% (Table 4). This degree of precision, far from ideal, may be useful in biological experiments in which the analysis of the positional dienoic isomers of biological interest is required, and in which the range of isomers is more restricted than in this study. It should be useful, especially in detecting major unexpected isomers.

On one hand, the present investigation was made feasible by the availability of pure synthetic isomeric octadecadienoates over a decade ago (4), permitting studies on known structures in mixture. On the other hand, progress was limited by the need for the development of techniques such as high-resolution CGC for the separation of aldehydes and the advent of computers in biological research. The development of CGC and computer procedures for matrix solution made the present project possible.

Using all, even redundant, information is an advantage of the matrix solution procedure. It provides for the least-squares minimization of all values entered. One disadvantage is the reporting of small proportions of isomers that may not be present. For example, the 5,10- and 7,10-isomers of Table 1 can be justified because A8, AE5, AE7, AA5 and AA3 were observed among the products of ozonolysis in Table 3. Another disadvantage is the need for eliminating minor aldehydic components from the input data to cause a matrix solution to converge. Thus, the solutions reached are approximations, presence or absence of minor components cannot be ascertained, and the quantification is less precise.

Comparison of results from the CGC of the initial methyl octadecadienoate mixtures with the results of the ozonization procedures shows that even with state-of-the-art CGC, only one of the seven synthetic isomer mixtures could be sufficiently resolved to be measured. Even two of the three isomers of 18:2 in the products of hydrazine reduction overlapped, giving an inflection point not always detectable as a binary peak. It is hardly likely that others of the potential 55 isomers to be encountered would be more separable. The ozonolysis-computer procedure is capable of estimating components in mixtures that cannot be resolved by present state-of-the-art CGC.

In the first use of our method on a biological sample (summarized only briefly here), unusual diene structures have been detected in liver PL. More comprehensive subsequent experiments confirmed and extended this observation (18). The unexpected dienes may be substrates for elongation and/or desaturation reactions in vivo to produce longer chain polyunsaturated fatty acids of unusual structure. In turn, these may be substrates for synthesis of eicosanoids of unusual structures and biological activities (3,16). This proposed method now will permit a more detailed study of the first step in the cascade of biological reactions that may have unpredictable and subtle effects upon metabolism.

The need for improving the precision of our preliminary analytical ozonolysis procedure is obvious. The yield of aldehydes by reductive-ozonolysis is not quantitative; it appears to vary with structure of dienoic fatty acid, whether mono- or dialdehyde is formed and, according to our unpublished kinetic studies, with degree of ozonization. These indications point the way for future improvements in our current methodology.

A measurement of the malondialdehyde peak has in the past been improved by a second injection 7- to 10-fold greater in size to amplify the minor peaks (2). Equivalent analyses for alpha methylene groups by nuclear magnetic resonance (NMR) and conjugatable dienes by alkaline isomerization (17), by lipoxygenase (17), or by injector port alkaline isomerization-gas chromatography (9) also are available. The latter procedure was employed in this study because it is easily performed and requires only a small sample, but it also needs improvement in precision.

In the future, the method needs to be extended to distinguish the *cis*- and *trans*-isomers. Hope that this can be achieved is found in the observation of the formation and CGC separation of *cis*- and *trans*-unsaturated aldehydes and aldehyde esters after partial ozonization. Information on the geometric configuration of the double bonds closest to the methyl group of the dienoic fatty acids would come from the configurations of the unsaturated aldehyde fragments, and on the double bonds closest to the ester end from the unsaturated aldehyde ester fragments.

The ozonization, CGC and computer matrix procedure at its present stage of development should be adaptable by minor modifications of program and procedure to the isomers of 20:2 that are known to occur in liver. The projected method should have broad applications to current analytical, biochemical and nutritional problems. These are the directions for our current biological studies.

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REFERENCES

1. Dutton, H.J. (1983) in *Dietary Fats and Health*, pp. 209-219, American Oil Chemists' Society, Champaign, IL.
2. Johnston, A.E., Dutton, H.J., Scholfield, C.R., and Butterfield, R.O. (1978) *J. Am. Oil Chem. Soc. 55*, 486-490.
3. Holman, R.T., and Mahfouz, M.M. (1981) *Prog. Lipid Res. 20*, 151-156.
4. Dutton, H.J., Johnson, S.B., Heimermann, W.H., Pusch, F.J., Holman, R.T., and Gunstone, F.D. (1985) in *Fat Science 1983. Proceedings of the 16th ISF Congress, Budapest, Hungary, October 4-5, 1983* (Hollo, N., ed.) Vols. 1 and 2, pp. 61-77, Elsevier Publishing Co., New York.
5. Gunstone, F.D., and Lie Ken Jie, M. (1970) *Chem. Phys. Lipids 4*, 1-14.
6. Bitner, E.D., Davison, V.L., and Dutton, H.J. (1969) *J. Am. Oil Chem. Soc. 46*, 113-117.
7. Bonner, W.A. (1953) *J. Chem. Educ. 30*, 452-453.
8. Stein, R.A., and Nicolaides, N. (1962) *J. Lipid Res. 3*, 476-480.
9. Bitner, E.D., Lanser, A.C., and Dutton, H.J. (1971) *J. Am. Oil Chem. Soc. 48*, 633-635.
10. Dutton, H.J. (1983) *J. Am. Oil Chem. Soc. 60*, 747.
11. Mahfouz, M., and Holman, R.T. (1980) *Lipids 15*, 63-65.
12. Harris, W.E., and Habgood, H.W. (1966) in *Programmed Temperature Gas Chromatography*, 142, John Wiley and Sons, New York.
13. Kleiman, R.F., Spencer, G.F., Earle, F.R., and Wolff, I.A. (1969) *Lipids 4*, 135-141.
14. Dal Nogare, S., and Juvet, R.S. Jr. (1962) in *Gas-Liquid Chromatography*, 220, Interscience, New York.
15. Sand, D., Sen, N., and Schlenk, H. (1965) *J. Am. Oil Chem. Soc. 42*, 511-516.
16. Mahfouz, M.M., Valicenti, A.J., and Holman, R.T. (1980) *Biochim. Biophys. Acta 618*, 1-12.
17. *Official and Tentative Methods of the American Oil Chemists' Society*, Am. Oil Chem. Soc., Champaign, IL, Methods Cd-5-40, 1-3 and Cd-15-78, 1-3.
18. Holman, R.T., Pusch, F., Svingen, B., Sand, D.M., and Dutton, H.J. (1987) *J. Am. Oil Chem. Soc. 64*, 649.

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Determination of Individual Long-chain Fatty Acyl-CoA Esters in Heart and Skeletal Muscle

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A method has been developed for determination of individual long-chain fatty acyl-CoA esters from heart and skeletal muscle using high performance liquid chromatography (HPLC). The esters were extracted from freeze-clamped tissue of pig and rat hearts and rat skeletal muscle for analysis on a radially compressed C_{18} 5μ reverse-phase column. Nine peaks in the extract with carbon chain lengths from C_{12} to C_{20} that subsequently disappeared on alkaline hydrolysis were identified. The major acyl-CoA peaks were 14:1, 18:2, 16:0 and 18:1 and additionally in rat heart 18:0. Total long-chain acyl-CoA esters obtained by summation of the individual molecular species was 11.34 ± 1.48 nmol/g wet wt. pig heart; 14.51 ± 2.11 nmol/g wet wt. in rat heart, and 4.35 ± 0.71 nmol/g wet wt. in rat skeletal muscle. These values were approximately 132% of those obtained using a separate procedure that measured total CoA by HPLC after alkaline hydrolysis of the esters. The described method demonstrates the quantitation of individual acyl-CoA species in muscle tissue. Therefore, it has a number of advantages in that it permits information to be obtained on the individual molecular species under various nutritional and metabolic conditions.
Lipids 23, 490–492 (1988).

Under normal physiological conditions, fatty acids are the preferred substrate for myocardial metabolism (1–3) and during long-term exercise are a major source of energy for skeletal muscle (4,5). As a result of myocardial hypoxia or ischemia, the mitochondrial oxidation of fatty acids in heart is impaired, and there is a consequent intracellular accumulation of long-chain fatty acyl-CoA and acylcarnitine esters (1,3,6). There have been few, if any, studies on acyl-CoA levels in skeletal muscle, and relatively little information is available on the metabolism of the individual esters in any intact tissue. A method for extraction of long-chain acyl-CoAs from rat liver and separation of the individual esters by high performance liquid chromatography (HPLC) has been developed (7). More recently, the technique has been used for the determination of long-chain acyl-CoAs in brown adipose tissue (8). This communication describes the adaptation of the procedure to heart and skeletal muscle, which contain considerably less long-chain acyl-CoA than liver (7). For control purposes, the sum of the individual long-chain acyl-CoAs was compared with their total concentration determined directly by alkaline hydrolysis and measurement of CoASH (9).

MATERIALS AND METHODS

Materials. Long-chain acyl-CoA esters for use as HPLC standards were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of the highest commercial grade possible.

Procurement and preparation of tissue samples. Eight adolescent swine weighing 45–70 kg were anesthetized with IV pentobarbitol (35 mg/kg), intubated with positive pressure ventilation using oxygen enriched room air (60–70% O_2) and the coronary arteries perfused via an arterial shunt connected extracorporally (10). Tissue samples of approximately 0.5 g were obtained at the completion of the two-hr aerobic procedure from the left anterior descending and left circumflex regions of the hearts. Rat hearts and total muscle from the hind limbs were removed as quickly as possible following the killing of 200–300 g animals maintained on Purina lab chow. Three to four hearts or muscles from both legs were pooled for single samples. All tissues were immediately frozen in liquid nitrogen and kept at −70 C until analyzed for long-chain fatty acyl-CoA esters.

Tissue long-chain acyl-CoA extraction. The procedure was based on that described previously for extraction of rat liver (7) with slight modification. Approximately 1–2 g of frozen tissue were powdered and suspended in 2 ml isopropanol and 2 ml 50 mM KH_2PO_4, pH 7.2, to extract the lipids as described by Mancha et al. (11) for a complex mixture of plant lipids. Free fatty acids and less polar lipids were removed by acidifying the suspension with 50 μl concentrated acetic acid and then extracted with petroleum ether saturated with 50% aqueous isopropanol. The long-chain acyl-CoA esters next were extracted with chloroform/methanol (1:2, v/v) after addition of 100 μl saturated $(NH_4)_2SO_4$ to the washed aqueous phase. The chloroform/methanol extract was roto-evaporated to dryness at 30–35 C.

To concentrate and purify the sample, a Waters C_{18} Sep-Pak was equilibrated with 2 ml methanol, 2 ml water and 2 ml 10 mM KH_2PO_4, pH 5.3. The sample was dissolved in 10 mM KH_2PO_4, pH 5.3 and applied to the Sep-pak. The Sep-pak was washed with 3 ml of 80% 10 mM KH_2PO_4 (pH 5.3)/20% acetonitrile. The sample was then eluted with 2 ml of buffer containing 30% 10 mM KH_2PO_4 (pH 5.3)/70% acetonitrile, followed by 2 ml of 75% methanol/% 10 mM KH_2PO_4, pH 5.3. The sample was evaporated to dryness with N_2, dissolved in 0.2 ml 10 mM KH_2PO_4/1 mM 2-Mercaptoethanol, pH 5.3, and injected into the HPLC system.

HPLC analysis of long-chain acyl-CoA. The analysis was carried out with a Waters Guard Column (2.3 cm length, 3.7 mm diameter) filled with a 37-50 Bondapak C-18 Corasil and a Waters Nova-Pak C_{18} reverse-phase (radial-pak cartridge, 10 cm length, 8 mm diameter). The two mobile phase solvents were A, 20% acetonitrile, 80% 10 mM KH_2PO_4, pH 5.3; and B, 70% acetonitrile, 30% 10 mM KH_2PO_4, pH 5.3. A slight modification of the linear gradient for elution of the long-chain acyl-CoA from that published (7) was divided into five steps. Step one was 92% solvent A reduced linearly to 72%, and 8% Solvent B increased linearly to 28% over a four-min period. Step two was 72% solvent A reduced linearly to 50%, and

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Abbreviation: HPLC, high performance liquid chromatography.

28% Solvent B increased linearly to 50% over a 10-min period. Step three was 50% solvent A reduced linearly to 25%, and 50% Solvent B increased linearly to 75% over a seven-min period. Step four was solvent A held at 25% and solvent B held at 75% for 10 min. Step five was 25% solvent A increased by curve 0.5 to 92%, and 75% Solvent B decreased by curve 0.5 to 8% over a 10-min period.

The volume of the sample injected was 200 μl, the flow rate 2 ml/min, total running time 41 min, and the equilibration time between runs was 10 min. The long-chain acyl-CoAs were detected at 254 nm at 0.016 absorbance units full-scale. Quantitation was based on peak areas, and excellent proportionality was obtained between the different volumes of the standard or extract injected and the concentrations of the individual esters. The equipment consisted of a Perkin Elmer Series 3B pump module, a variable wavelength detector with an autocontroller, a Sigma 15 data processor, and a Waters WISP 710 B automatic sample injector.

As a check to this method, total long-chain acyl-CoA also was determined by an independent HPLC method of Ingebretsen et al. (9) and modified in this laboratory for heart tissue. The CoA esters were extracted from pulverized frozen tissue by perchloric acid precipitation and assayed for free CoASH released by alkaline hydrolysis. To prevent oxidation, 20 mM 2-mercaptoethanol was added to the extracts throughout the base hydrolysis. Tissue samples were compared with acyl-CoA standards by co-chromatography using a Waters Partisil SAX (strong anion exchange) Radial-PAK cartridge (8 mm × 10 cm). Elution was isocratic using a solvent composition of thiodiglycol/2-propanol/196 mM phosphate buffer, pH 3.9 (0.05:2.0:97.95). The flow rate was 2 ml/min, and detection was at 260 nm. Sample recoveries of 95% were estimated by the addition of known amounts of palmitoyl-CoA to aliquots of tissue samples. Total tissue acyl-CoA content was compared with the sum of the individual esters separated above.

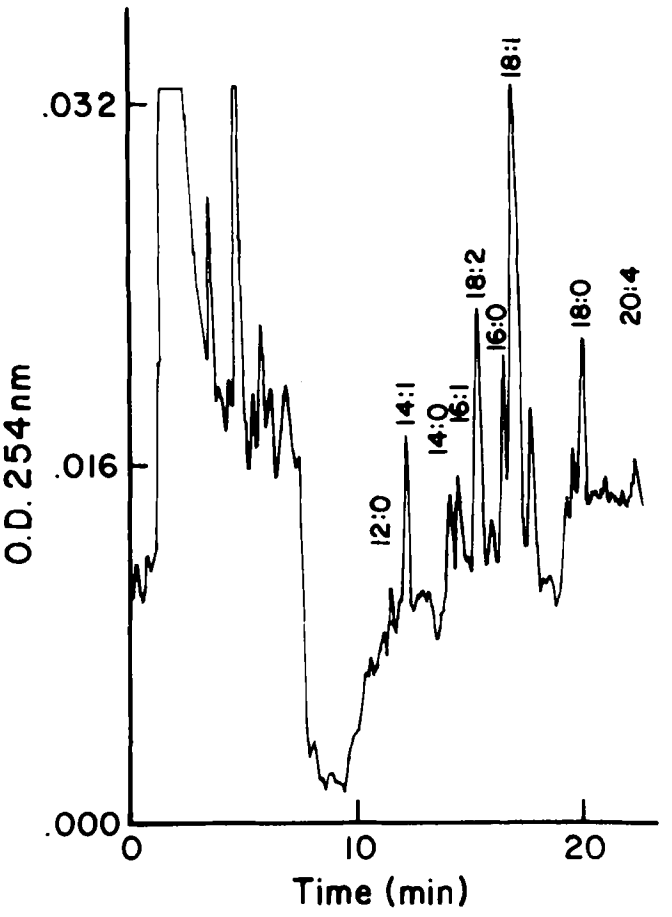

FIG. 1. HPLC separation of long-chain acyl-CoA esters from extracts of frozen powdered pig hearts. The procedure was carried out as described in Materials and Methods.

RESULTS

In a previous report (7), the chromatographic separation of a mixture of eight standard long-chain fatty acyl-CoA esters was demonstrated and verified by their disappearance following alkaline hydrolysis. More recently, the elution of added arachidonyl-CoA has extended the number to nine. This is a noteworthy addition to the procedure because in many tissues, arachidonic acid and its CoA thioester are substrates for a number of important enzymatic reactions, particularly in phospholipid and prostaglandin synthesis.

A typical separation pattern of long-chain acyl-CoA esters obtained from an extract of frozen pig heart tissue is shown in Figure 1. Although the baseline is somewhat skewed, possibly due to concentration of the extract, nine peaks in the order of increasing retention times easily were identified and quantified. These corresponded to the acyl CoAs 12:0, 14:1, 14:0, 16:1, 18:2, 16:0, 18:1, 18:0 and 20:4. The peak eluting just after 18:1 cannot be identified yet because it does not correspond to any of the available standards. Over 85% of radioactive palmitoyl and oleoyl CoA added before the chloroform/methanol extraction could be recovered.

Table 1 shows the concentrations of the individual long-chain acyl-CoA esters obtained from samples of pig heart, rat heart and rat skeletal muscle. Myristoleoyl-CoA (14:1), linoleoyl-CoA (18:2), palmitoyl-CoA (16:0) and oleoyl-CoA (18:1) were in the highest concentrations. In most samples, the amounts of lauryl (12:0) and arachidonyl (20:4) CoA were barely detectable. There essentially was no difference in the values from the pig heart tissue sites perfused by the left anterior descending and left circumflex arteries. The sum of the individual fatty acyl-CoA ester values was equivalent to approximately 132% of their concentration obtained from the separate HPLC analysis of total CoASH following alkaline hydrolysis of the esters (9). The two methods thus compare quite favorably. This latter method (9), which frequently has been frequently used and referred to in the literature, was specifically adapted for use in heart tissue for these respective experiments.

Rat and pig heart total and individual acyl-CoA esters are quite similar except for the relatively higher level of stearoyl-CoA in rat heart. Other than oleoyl-CoA, rat skeletal muscle contains considerably less acyl-CoA than heart. The quantity of long-chain acyl-CoA in heart and skeletal muscle is considerably less than that in liver requiring modifications in preparation of the tissue for

TABLE 1

Long-chain Acyl-CoA Levels in Pig Heart, Rat Heart and Rat Skeletal Muscle

Acyl-CoA	nmol/g wet wt.		
	Pig heart	Rat heart	Rat skeletal muscle
12:0	0.46 ± 0.26	0.94 ± 0.77	0.09 ± 0.06
14:1	1.62 ± 0.53	1.97 ± 0.86	0.09 ± 0.09
14:0	0.80 ± 0.36	0.76 ± 0.42	0.19 ± 0.07
16:1	0.87 ± 0.40	0.53 ± 0.46	0.24 ± 0.06
18:2	1.63 ± 0.37	1.20 ± 0.99	0.73 ± 0.11
16:0	1.78 ± 0.29	1.96 ± 0.81	0.87 ± 0.29
18:1	2.84 ± 0.59	2.10 ± 0.44	1.33 ± 0.41
18:0	0.92 ± 0.26	4.73 ± 1.54	0.71 ± 0.46
20:4	0.42 ± 0.48	0.32 ± 0.27	0.10 ± 0.14
Total	11.34 ± 1.48	14.51 ± 2.11	4.35 ± 0.71

HPLC analysis. In particular, the extract must be concentrated before separation and detection of the esters, which can result in some background noise. Use of the C_{18} Sep-Pak column for this purpose also removes a considerable amount of contaminants and facilitates concentrating the samples. This results in an increase in the peak heights relative to the baseline, thereby enhancing the reproducibility. Washing the column with buffer at the end of the run and running steps 4 and 5 for 15 rather than 10 min, if the baseline begins to rise at the end of the run, leads to a more stable baseline that is consistent from run-to-run. The minimal standard deviations of the values shown in Table 1 indicate that reproducibility is not a significant problem except for quantitation of the minor peaks.

DISCUSSION

The importance of fatty acid metabolism in overall cardiac and skeletal muscle performance is well-appreciated, although its potential role in pathophysiological states such as myocardial ischemia is not fully agreed upon and, in fact, is somewhat controversial (1,3,6,12-16). There is a considerable amount of detailed information on alterations of total free fatty acids as well as their acyl-CoA and acylcarnitine esters in the myocardium under different physiological and pathological conditions (6,10,12, 15,16). However, virtually no information is available for skeletal muscle. This study demonstrates that it now is feasible to obtain by direct analysis, a quantitative measurement of individual long-chain fatty acyl-CoA esters. Thus, it should be possible to determine their individual metabolic fluxes for different experimental conditions. Moreover, the method has been modified slightly to include separation and measurement of arachindonyl-CoA whose concentration might reflect the metabolism of arachidonic acid into various pathways including phospholipid and prostaglandin synthesis, which are very important in myocardial and skeletal muscle function.

The method described in this communication, which is relatively simple, should be of particular value for metabolic studies.

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REFERENCES

1. Neely, J.R., and Morgan, H.E. (1974) *Ann. Rev. Physiol. 36,* 413-459.
2. Williamson, J.R. (1979) *Ann. Rev. Physiol. 41,* 485-506.
3. Corr, P.B., Gross, R.W., and Sobel, B.E. (1984) *Cir. Res. 55,* 135-154.
4. Havel, R.J., Narmark, A., and Borchgrevink, C.F. (1963) *J. Clin. Invest. 42,* 1054-1063.
5. Issekutz, B. Jr., Paul, P., and Miller, H.I. (1967) *Am. J. Physiol. 213,* 857-862.
6. Idell-Wenger, J.A., Grotyohann, L.W., and Neely, J.R. (1978) *J. Biol. Chem. 253,* 4310-4318.
7. Woldegiorgis, G., Spennetta, T., Corkey, B.E., Williamson, J.R., and Shrago, E. (1985) *Anal. Biochem. 150,* 8-12.
8. Donatello, S., Spennetta, T., Strieleman, P., Woldegiorgis, G., and Shrago, E. (1988) *Am. J. Physiol. 254,* E181-E186.
9. Ingebretsen, O.C., Normann, P.T., and Flatmark, T. (1979) *Anal. Biochem. 96,* 181-188.
10. Liedtke, A.J., Nelles, S.H., and Mjos, O.D. (1984) *Am. J. Physiol. 247,* H367-H394.
11. Mancha, M., Stokes, J.B., and Stumpf, P.K. (1975) *Anal. Biochem. 68,* 600-608.
12. Shug, A.L., Shrago, E., Bittar, N., Folts, J.D., and Koke, J.R. (1975) *Am. J. Physiol. 228,* 689-692.
13. Shrago, E., Shug, A.L., Sul, H., Bittar, N., and Folts, J.D. (1976) *Circ. Res. 38,* Suppl. 1, 75-78.
14. Opie, L.H. (1979) *Am. Heart J. 97,* 375-388.
15. Moore, K.H., Radloff, J.F., Hull, F.E., and Sweeley, C.C. (1980) *Am. J. Physiol. 239,* H257-H265.
16. Hulsmann, W.C., Stam, H., and Maccari, F. (1982) *Biochim. Biophys. Acta 713,* 39-45.

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Nanogram Quantification of Nonpolar Lipid Classes in Environmental Samples by High Performance Thin Layer Chromatography

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A sensitive, simple, and rapid high performance thin layer chromatography (HPTLC) method that quantifies nanogram amounts of complex nonpolar lipid classes in environmental samples with a minimum of sample preparation is presented. The derivatization method is lipid-specific and insensitive to carbon number or the degree of unsaturation of the fatty acids composing the lipid class compounds. Nonpolar lipid classes ranging from 10 to over 500 ng easily may be quantified in the same sample run. The coefficient of variation for sample replicates on different plates ranged from 2.3% to 5.9%. Accuracy of the method is better than 15%. The nonpolar lipid class composition of <53 μm oceanic particles in a vertical depth profile of the upper 600 meters of the Slope Water was determined to illustrate the HPTLC method. The observed changes in particulate lipid class composition indicate that secondary production by deep-living organisms significantly alters suspended particle composition with depth in the water column.
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Lipid classes are useful tracers of the sources and transformations of organic carbon in particulate material in the water column (1-4), sediments (5-7) and other environmental materials. However, complete characterization of the lipid classes in these samples has been limited by the lack of simple methods to resolve complex mixtures of plant, animal and microbial lipids, the time required for analyses by existing methodologies, and the small amount of material often available. In our studies of oceanic particulate material, we required an analytical technique that could adequately resolve and quantify sub-μg amounts of complex lipid classes yet was simple enough for routine analyses. No existing method satisfied these requirements.

Ideally, a method used to resolve and quantify complex lipid class composition in such small environmental samples should be very sensitive yet require a minimum amount of sample manipulation. Previous studies have used either column (1-4) or thin layer chromatography (5,7-9) to separate lipid classes, followed by gravimetric, spectrophotometric or gas chromatographic analyses to quantify the lipid fractions. These methods are tedious and time-consuming and allow only a few samples to be processed per day. The multiple steps involved can result in sample loss, fractionation or degradation. Moreover, complex lipid classes often overlap in the fractions so additional steps are required to purify each lipid class. Tens to hundreds of μg of total lipids may be needed to accurately quantify minor lipid classes in a complex sample by these methods. High performance liquid chromatography (HPLC) has been used to separate lipid classes (10,11), but quantitative measurement of the nonpolar lipid classes has not been achieved. Alternatively, lipid classes have been separated on thin-layer plates and derivatized in situ to form compounds that can be quantified by scanning densitometry (6,8,12-14). This method is more expedient than the above methods but requires μg amounts of lipid classes and has limited precision due to incomplete separation of complex lipid classes and high background noise. More recently, the Iatroscan/Chromarod system has been used to determine the lipid class composition in a variety of materials (15-21). While the Iatroscan/Chromarod has found widespread use in routine lipid analysis, the system has limitations in sub-μg analyses of complex lipids due to problems with nonreproducibility, poor precision, lack of sensitivity and nonlinearity of response (21-23). None of these techniques were sufficiently sensitive and expedient for routine analyses of lipid class composition in our samples.

However, recent advances in quantitative high performance thin layer chromatography (HPTLC) suggested that this method might be ideally suited for sub-μg quantification of complex lipid classes. Quantitative HPTLC combines the many inherent advantages of TLC with high resolution and subnanogram detection limits for a wide variety of compounds (24,25). The newly developed preadsorbent sample application zone on HPTLC plates eliminates the sample application difficulties previously encountered in HPTLC and serves to cleanup and narrowly focus the sample at the preadsorbent zone/silica gel interface, thus enhancing the separation of complex lipids (26). The development of fluorescent-inducing reagents (27-33) has enabled nanogram quantities of the major lipid classes to be detected easily in samples of uniform biological origin such as blood serum, amniotic fluid and tissue extracts (33-36). This study investigated whether HPTLC methods also were suitable for quantitative analyses of complex lipid classes of unknown composition in environmental samples.

In this paper, we show that HPTLC is a rapid, sensitive, quantitative and reproducible technique for sub-μg analyses of complex lipid classes in oceanic particulate material. The HPTLC method presented here generally is applicable to most materials. The resolution of complex lipid classes is more complete, and the optimal concentrations for quantitative work are nearly two orders of magnitude less with HPTLC than with currently employed methods, including the Iatroscan/Chromarod system. In addition, the method is very rapid and allows up to 50 analyses of crude lipid extracts to be easily completed per day.

METHODS

Materials and reagents. HPTLC silica gel plates (10 × 10 cm) with preadsorbent sample application zones were

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Abbreviations: MG, monoacylglycerol; DGE, diacylglycerol ether; DG, diacylglycerol; CF, cholesteryl formate; FAL, fatty alcohol; FAME, fatty acid methyl ester; FFA, free fatty acid; POC, particulate organic carbon; TG, triacylglycerol; ST, sterol; STE, steryl ester; WE, wax ester; FID, flame ionization detector; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; TLC, thin layer chromatography.

obtained from Merck (Darmstadt, FRG). Lipid class standards were obtained from Nu-Chek Prep, Inc. (Elysian, MN). Free fatty acids were obtained from Sigma Chemical Co. (St. Louis, MO). Aminopropyl Bond-Elut disposable chromatography columns with stainless steel frits were used for preparative separation of lipid classes and were obtained from Analytichem International (Harbor City, CA). Prechanneled thin layer chromatography (TLC) plates with preadsorbent zones were used for purification of the lipid class fractions and were obtained from Whatman (Clifton, NJ). All solvents were chromatography grade.

Sample collection and preparation. Oceanic particulate material was collected from the upper 1000 meters using large-volume in situ fitration (37). Samples described in this paper are from the Slope Water 200 km off the coast of Virginia and were collected in June 1982. Further details of the collection method and the sample site can be found in Bishop et al. (38).

The filter samples were subsampled within two hr of retrieval. Each subsample represented about 10% of the total material collected by filtration and contained approximately 2 to 5 mg of organic carbon. Subsamples were placed in 20-ml test tubes and filled to the top with chloroform/methanol (2:1, v/v). The antioxidant BHT (0.05%, w/v) was added to the sample tubes approximately six months later. Samples were stored at −20 C until analyzed. There was no evidence of compound oxidation or hydrolysis, or fatty acid methyl ester formation in the samples during prolonged storage.

Lipids were extracted using a modification of the procedure described by Folch et al. (39). Cholesteryl formate was added as an internal TLC standard before extraction to correct for sample losses and spotting variability. The chloroform/methanol solvent in the sample tubes was vacuum-filtered through a fritted funnel into a separatory funnel, and the sample tube was refilled with fresh chloroform/methanol (2:1, v/v). The sample tube then was ultrasonicated in a cup horn at 12 C at 100 watts for 45 sec using a Sonifier cell disruptor (Heat Systems-Ultrasonics, Plainview, NY). This solvent then was filtered through the fritted funnel into the separatory funnel and the filters rinsed with solvent. Aqueous KCl (0.88%, w/v) (1/4 the volume of the total solvent) then was added to the separatory funnel and the lipids extracted into the lower organic phase. The lower phase then was rotary evaporated at 30 C to the point of dryness, redissolved in chloroform and passed through a mini-cleanup column containing sodium sulfate to remove any residual water. The final volume of the crude lipid extract then was adjusted to 2 ml with chloroform.

Standard lipid classes. Simple lipid class mixtures comprised of monoolein (MG), diolein (DG), triolein (TG), oleic acid (FFA), methyl oleate (FAME), stearyl oleate (WE), cholesterol (ST) and cholesteryl oleate (STE) were prepared in concentrations ranging from 1 to 160 ng/μl. The internal standard cholesteryl formate also was added to these standard mixtures to correct for spotting variability and evaporation. The amount of cholesteryl formate added to each standard was adjusted to produce an average final concentration of 50 ng per 10 μl spot.

Complex lipid classes purified from a larger sample of oceanic particulate material were used to calibrate the response of simple lipid class standards with the complex lipid classes found in samples. The lipid classes in the oceanic particulate material were separated using disposable aminopropyl Bond-Elut chromatography columns (40). The lipid class fractions then were purified by TLC using hexane/ether/acetic acid (80:20:1, v/v/v) as the developing solvent.

These sample lipid classes were independently quantified by capillary gas chromatography. The free fatty acid and triacylglycerol fractions were transesterified with 5% methanolic HCl (41) at 50 C for 12 hr to form fatty acid methyl esters. Nonadecanoic acid was added as an internal standard before transesterification. The fatty acid methyl esters were analyzed using a Carlo Erba 5160 gas chromatograph equipped with on-column injection. Chromatography was performed on a 50 m × 0.25 mm CP Sil 88 column (Chrompack, Bridgewater, NJ) programmed from 110 to 220 C at 5 C/min. The GC was interfaced to an Apple IIe computer using a SOFT 510 system (Data Acquisition Systems, Boston, MA) and peaks were quantified using DYSC chromatography software (Dynamic Solutions Corp., Pasadena, CA). The TG concentration was corrected to include the additional weight of the glycerol moiety.

Thin layer chromatography. HPTLC plates were predeveloped to remove impurities and binder material that impart a high background fluorescence. Plates first were predeveloped in methanol and then in the same polar solvent later used for development (methyl acetate/n-propanol/chloroform/methanol/43 mmol/l aqueous potassium chloride, 25:25:25:10:9, v/v/v/v/v) (35). A thin line was then scraped across the top of the plate about 3 mm from the top to prevent these impurities from back-diffusing into the upper part of the plate during later developments. The plates were then dried at room temperature and activated in a 110 C oven for one hr before chromatography. Plates were handled carefully to maintain a dust-free, unmarred surface.

Seven samples and two standard mixtures were applied to each 10 × 10 cm plate. The lipid class concentrations of the two standard mixtures bracketed the expected lipid class concentrations in the samples. Either 5 or 10 μl of lipid extract or standards were applied quantitatively to the preadsorbent zone about 5 mm from the end of the plate using an Oxford 5 μl fixed volume micropipette with a Teflon tip (Sherwood Medical, St. Louis, MO). This volume can be rapidly yet quantitatively applied to the preadsorbent zone of HPTLC plates and eliminated the need for a sample concentration step. Each sample was applied as a line of overlapping spots perpendicular to the preadsorbent interface and parallel to the direction of development. Spot diameter was maintained at <4 mm. This application method enables the sample lipids to be focused in a narrow band at the preadsorbent interface at the beginning of development. A gentle stream of nitrogen was used to evaporate the excess solvent during application. Although this application method results in slightly higher sample concentrations at the edges of the sample band than in the center, the uneven distribution has negligible effect on quantitation provided the width of the sample band is less than the slit height used for densitometry so that the entire sample band is scanned.

Plates were developed in saturated TLC tanks by one of two methods. Method I: the plate was developed in

hexane/heptane/ether/acetic acid (63:18.5:1, v/v/v/v) (35) for 10 min (6 cm), dried with a stream of unheated air using a hairdryer for two min, then developed twice in hexane/benzene (3:1, v/v) for 15 min.

While this developing method gave good resolution, the silica gel surface near the preadsorbent zone interface frequently was uneven, which caused problems with quantification by scanning densitometry. In addition, development by Method I did not completely separate the internal standard from the FAME region and wax esters from the steryl ester region. Therefore, later experiments used a different developing scheme (Method II) that moved the nonpolar lipids farther away from the preadsorbent zone interface and increased the resolution between the lipid classes. We also found Method II gave slightly lower backgrounds.

Method II: this method used a series of three developing solvents. The plate was first developed in carbon tetrachloride for eight min (5 cm) to narrowly band the sample lipids at the preadsorbent/silica gel interface and to remove the antioxidant BHT, which migrates with the solvent front, away from the sample zone. Next the plate was developed in methyl acetate/1-propanol/chloroform/methanol/43 mmol/1 KCl (25:25:25:10:9, v/v/v/v/v) for two min (0.4 cm) to separate the neutral lipids from the polar lipids and to remove the neutral lipids from the interface zone. The neutral lipids then were separated using double development in hexane/heptane/ether/acetic acid (63:18.5:18.5:1, v/v/v/v) (35) for eight min (6.5 cm). This double development increased the resolution of the lipid classes. Finally, the plate was developed in carbon tetrachloride for 30 min to more completely separate the internal standard cholesterol formate from fatty acid methyl esters, and steryl esters from wax esters. The plate was dried under a stream of unheated air using a hairdryer for two min between developments.

Derivatization. The lipid classes were derivatized using the method of Schmitz et al. (35). The developed plate was thoroughly air-dried and then dipped in the manganese chloride-sulfuric acid derivatizing reagent (3.2 g $MnCl_2$, 32 ml conc. H_2SO_4, 480 ml H_2O, 480 ml methanol) (27) for 20 sec. The back of the plate then was blotted to remove excess reagent and the plate dried with a cold hairdryer for five min to evaporate the solvents. The plate was placed in a 110 C oven for exactly 40 min to form fluorescent derivatives of the lipid classes. Following derivatization, plates were stored in a dessicator until scanned.

Densitometry. Plates were scanned using a Camag II scanning densitometer (Camag, Inc., Wrightsville Beach, NC) in the reflectance mode. The monochromator on the mercury light source was set for an excitation wavelength of 366 nm and 10 nm bandwidth. A 400 nm emission filter was used. Slit dimensions were 0.2 mm width by 6 mm height, so that the slit height completely covered the width of the sample lane. A scanning speed of 0.5 mm/sec was used. Sensitivity was adjusted so that the maximum peak on the plate was near 100% full-scale response of the densitometer.

Peaks were integrated using a SP 4270 integrator (Spectra-Physics, San Jose, CA). Baseline irregularity, incomplete peak resolution and the irregular peak shapes of some complex lipid classes, such as the triacylglycerols and wax esters, often limited the ability of this integrator to accurately detect and quantify compound peaks. More sophisticated peak detection and integration systems (such as the DAS/DYSC system used for GC analyses) should be used to improve the accuracy of baseline detection and peak quantification.

RESULTS AND DISCUSSION

A developed HPTLC plate illustrating the separation of nonpolar lipid classes in simple lipid class standard mixtures and samples of oceanic particles is shown in Figure 1. The major nonplar lipids are well separated above the preadsorbent zone/silica gel interface. The internal standard cholesteryl formate (CF) is well separated from the sample lipid classes. Monoacylglycerols, glycolipids and polar lipids remain at the interface. Note that while the standard lipid classes that are comprised of a single compound migrate in a tight band, the complex sample

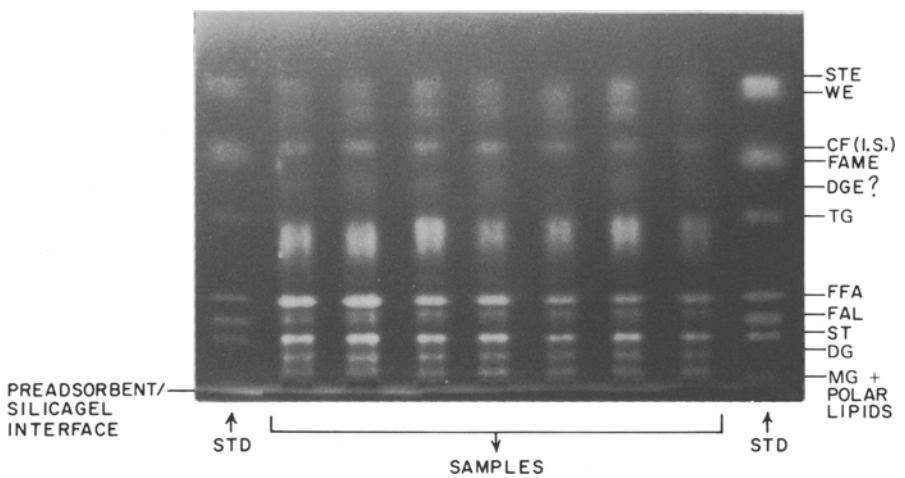

FIG. 1. Developed HPTLC plate showing the separation of nonpolar lipid classes in simple lipid standard mixtures and in samples of oceanic particles from a Slope Water profile taken in June 1982. Development was by Method I, as described in the text. The plate is viewed under UV light and has been overloaded for photographic purposes.

lipid classes migrate in a wide band due to the differences in relative mobilities of the fatty acids composing the lipid class compounds. This effect is especially pronounced for triacylglycerols and wax esters. The fluorescent response is lipid-specific; pigments do not react. In addition, the fluorescence of the different lipid classes show slight variations in color that aid in preliminary identification of sample unknowns.

Although much effort was made to standardize all aspects of the chromatography, variability in compound and background fluorescence often was significant, especially between plates run on separate days. Part of the background fluorescence could be attributed to impurities left in the plate or adsorbed following predevelopment. Lower backgrounds were obtained if the plate was predeveloped just before oven activation and use. Compound response and background fluorescence also is affected by the amount of residual solvent left on the plate. Some residual solvent is necessary for the derivatization reaction (a completely dried plate showed lower compound fluorescence) but too much solvent resulted in a high background. In addition, Schmitz et al. (35) found the development of fluorescence to be strongly time- and temperature-dependent. Therefore, slight differences in heating also may have been an additional source of variation. It is important to note that this interplate variability does not pose a problem in quantification because standards are run on the same plate as samples.

Much of the nonspecific background fluorescence diminished within the first few days, while compound fluorescence remained stable. Better results may be obtained if plates are allowed to sit dessicated for several days before scanning. No loss of sensitivity was detected when plates were rescanned after two months of dessicated storage. Therefore, plates can be stored for extended periods and easily rescanned or reintegrated to optimize quantification without rerunning the sample.

Standard curves. Standard curves demonstrate that nonpolar lipid classes varying by two orders of magnitude may be quantified on the same run. Response vs amount for simple lipid classes were constructed over a range of 5 to 300–700 ng. Two plates with standard lipid mixtures were run.

The first plate covered the range of standard lipid classes from 20–30 to 500–700 ng and was developed by Method I (Fig. 2). Response was proportional to the log of amount throughout the entire range for all classes except for cholesteryl oleate and methyl oleate, which showed linear relationships at concentrations below 100 ng. Standard curves for these compounds thus were split into two segments; a linear fit was used at low levels and a log linear fit at higher levels. Correlation coefficients better than 0.998 for all regression equations except the linear fit of cholesteryl oleate at concentrations <75 ng ($r^2 = 0.982$).

The second plate covered lower concentrations ranging from 5 to 200 ng. This plate was developed by Method II. Background fluorescence was significantly lower than on the first plate. The absolute limit of detection was 5 ng or less for all classes except for triacylglycerol, in which the limit of detection was 10 ng. Plate surface irregularity was the major factor limiting the ability to identify the sample peaks at the lowest levels. Response was a linear function of amount in the range of 5–120 ng

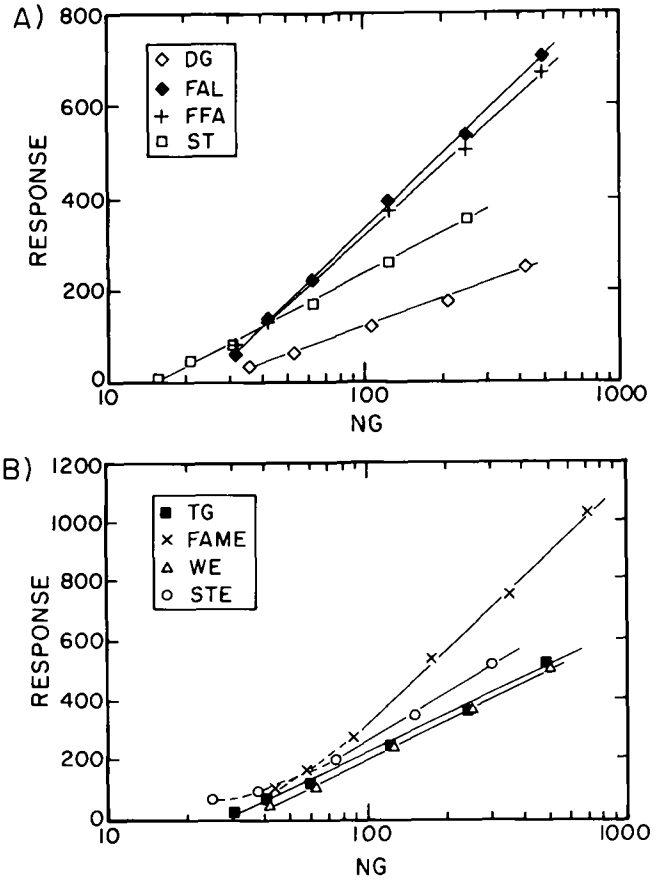

FIG. 2. Standard curves of fluorescent response vs log concentration for simple lipid classes on plate #1. Development was by Method I. Response is in arbitrary units. A) Standard curves for 1,3 diolein, cholesterol, oleyl alcohol and oleic acid; B) standard curves for triolein, methyl oleate, stearyl oleate and cholesteryl oleate.

(Fig. 3). However, response at 200 ng was significantly below that predicted from the linear standard curve, indicating the development of fluorescent quenching. Correlation coefficients of linear regression equations for 5–120 ng were over 0.996 for each standard.

In addition to the shapes of the standard curves, the relative responses of the various lipid classes also differed significantly between the two plates. Cholesterol and oleic acid, in particular, had much lower relative responses on the first plate. Schmitz et al. (35) found that fluorescence of the lipid derivatives increases with time to a maximum and then falls to zero as the derivatives ash. This decrease was found to be more rapid with cholesterol than with triacylglycerol. It is likely that the lower relative response of cholesterol and oleic acid on the first plate may have been due to partial ashing of their derivatives, which suggests that the timecourse of derivatization reactions also is influenced by conditions on the plate itself. As acidic conditions are necessary for fluorescence development, it is possible that slightly higher residual acid on the first plate due to differences in development may have influenced the reaction rates as well as the maximum fluorescence developed. Standardized conditions would reduce the interplate variability observed here. The highly significant standard curves demonstrate that excellent

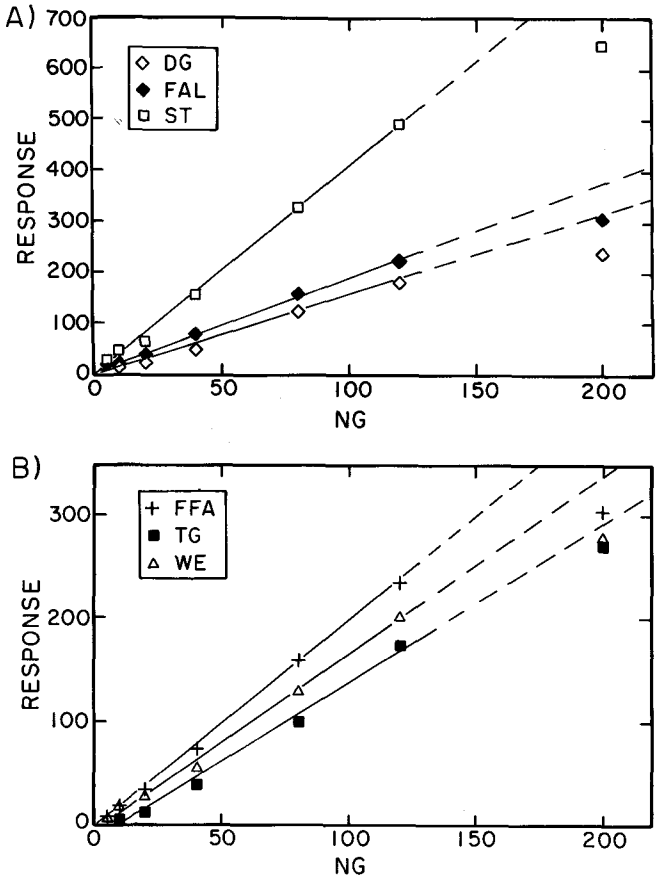

FIG. 3. Standard curves of fluorescent response vs concentration for simple lipid classes on plate #2. Development was by Method II, as described in the text. Response is in arbitrary units. Note the deviation from linearity of standard response at 200 ng, indicating the development of fluorescent quenching. A) Standard curves for 1,3 diolein, cholesterol and oleyl alcohol; B) standard curves for oleic acid, triolein and stearyl oleate.

quantitative results can be obtained throughout a wide range of reaction conditions.

The transition from a linear fit at tens of nanograms concentration to log linear fit at higher concentrations indicates that there is a threshold fluorescent density on the plate above which quenching of the response occurs. This transition from a linear to nonlinear relationship was also observed for lecithin and sphingomyelin derivatives formed by the reaction with HNO_3 vapors (36). Thus, the quenching response may be similar in all fluorescent derivatives, independent of the exact fluorescent inducing reagent.

Differences between the standard curves on the two plates indicate that the exact compound density at which this fluorescence threshold is first observed can vary significantly among plates. The first plate, which had a much higher background, exhibited nonlinearity at a much lower compound density. This suggests that quenching is a function of the overall background fluorescence of the plate as well as the total development of compound fluorescence during the derivatization reaction. These factors may explain the differences in the response vs concentration curves found in this study from that found by Schmitz et al. (35). Thus, external standards must be run on the same plate and in the same concentration range as sample compounds for accurate quantification. Because the concentration range at which nonlinearity first is observed differs from plate to plate, it is advisable to run several external standards if a wide range of sample concentrations is expected. For this purpose, a 10 × 20 cm HPTLC plate would allow both a complete standard curve and multiple samples to be developed simultaneously.

Calibration of sample lipid classes with standards. If standard lipid classes, which are comprised of only one compound, are to be used to quantify sample lipid classes, which are comprised of many different compounds, fluorescent response must depend solely on the concentration of lipid class present and not on the exact fatty acid composition of the lipid class. Two experiments were run to test for differences in response between simple liipid class standards and the real complex lipid classes encountered in samples.

The first experiment demonstrated that fluorescence of derivatives is relatively insensitive to both the carbon number and the degree of unsaturation of the fatty acid constituents (Fig. 4). Six fatty acids, ranging from 18 to 22 carbons and from one to six double bonds, were developed on the same plate, and the total responses of 100 ng of each acid derivative determined. No systematic trend of absolute response with either carbon number or number of double bonds was found at this compound density. However, the coefficient of variation among the different acids on the plate was 13.5%, nearly three times that found for the coefficient of variation of sample replicates on different plates. If this variation, in fact, was due to different fatty acid responses, it implies about a 15% accuracy in quantification at this compound density when simple lipid class standards are used. We did not investigate whether this insensitivity to degree of unsaturation varied with compound density on the plate. However, results of this experiment indicate that the $MnCl_2$ derivatives are less sensitive to lipid class composition than other fluorescent derivatives that show a dependence of fluorescence on component fatty acid unsaturation (34,42,43).

Sample lipid classes also differ from standards in peak geometry on the developed plate. As seen in Figure 1, lipid standards comprised of only one compound migrate in a narrow concentrated zone during development and have well-defined peak shapes. In contrast, complex lipid classes migrate in a wide sample band due to the different relative mobilities of the saturated and highly unsaturated fatty acid constituents of the lipid class compounds. Band broadening alters the compound density of the sample lipid classes on the plate from that of standards; this may significantly affect the amount of fluorescent quenching for a given concentration.

A second experiment demonstrated that these differences in peak shape do not significantly affect the quantification of sample lipid classes (Fig. 5). Fatty acids and triacylglycerols were purified from a large sample of coeanic particulate material. Subsamples from each lipid class were transesterified and then quantified as FAME by capillary gas chromatography to independently determine the concentrations of the sample lipid classes. Three amounts of each sample class, spanning the amounts usually found in samples, were applied to a plate. The

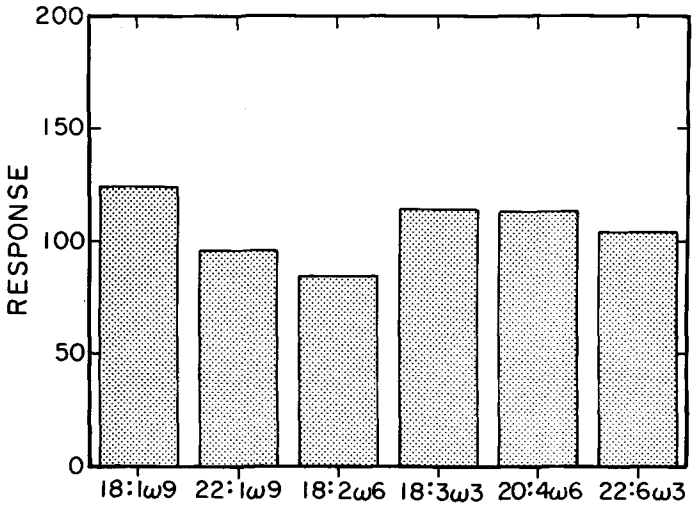

FIG. 4. Relative fluorescent response of 100 ng each of six fatty acids differing in both carbon number and number of double bonds. Development was by Method II. The coefficient of variation was 13.5%. There is no systematic trend of response with either chain length or degree of unsaturation.

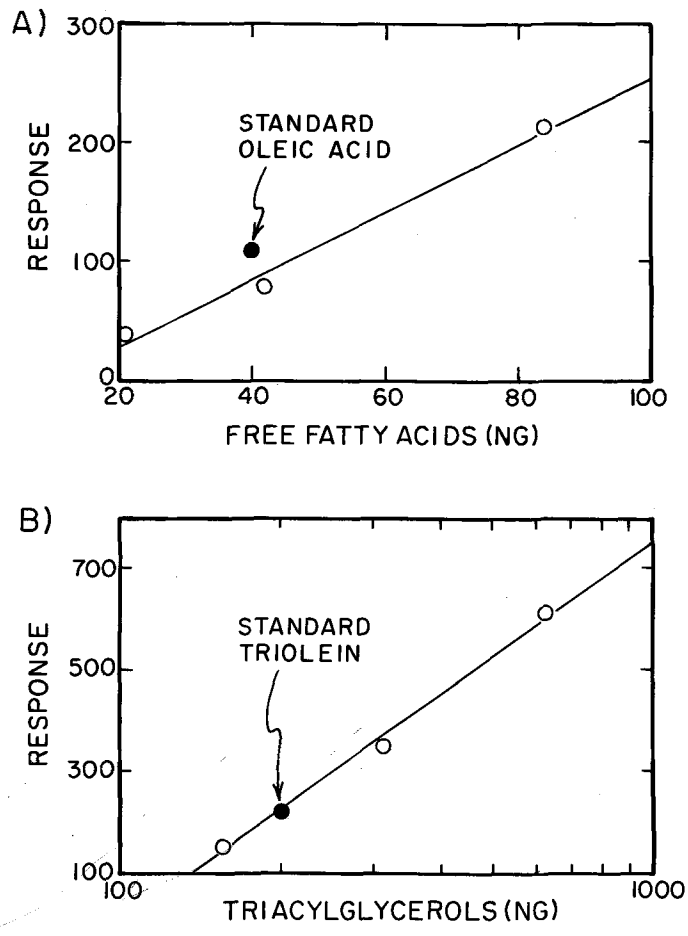

FIG. 5. Calibration of simple lipid standards with complex lipid classes purified from oceanic particulate material. Development was by Method II. The response of simple lipid standards is plotted for comparison against the regression curves calculated for the sample complex lipid classes. A) Fatty acids; B) triacylglycerols.

simple lipid standards oleic acid (FFA) and triolein (TG) were spotted in separate lanes. Regression curves were calculated for the sample lipids using a linear fit for free fatty acids (20–90 ng range) and a log linear fit for TG (150–600 ng range). Estimated amounts of the simple standards oleic acid and triolein calculated using the sample lipid class regression equations were compared with the actual amounts spotted. The standard oleic acid amount was estimated at 46 ng, within 15% of the actual amount of 40 ng spotted. The standard triolein amount was estimated at 198 ng, in excellent agreement with the actual amount of 200 ng. These results demonstrate that although slight differences in fluorescence may exist between simple lipid classes and the complex lipid classes found in samples, standards comprised of a single fatty acid constituent can be used to quantify complex lipid classes if standard and sample concentrations are in the same range. Alternatively, complex lipid mixtures approximating sample lipid class compositions could be used for more accurate quantification.

Application to samples of oceanic particulate material. Oceanic particulate material is an extremely complex environmental sample with plant, animal and microbial sources. There is a wide range of highly unsaturated fatty acids present in the acyl lipid classes. The demands of resolving this complex mixture and the large differences in class concentrations make the determination of lipid class composition in oceanic particles among the most difficult of lipid class analyses.

The HPTLC method was used to resolve and quantify the nonpolar lipid classes in a verical depth profile of <53 μm sized oceanic particulate material from the upper 600 meters of the Slope Water in June 1982. A representative chromatogram from a sample at 450 meters is shown in Figure 6. Major lipid classes were sterol, fatty alcohol, free fatty acid, TG, wax ester and a compound tentatively identified as DG. Another minor unidentified lipid, with a R_f similar to that of diacylglycerol ether (DGE) (44), was found just above the TG on the plate. FAME and STE were present only in trace amounts in these samples.

Lipid classes had a wide range of concentration in the same sample. Amounts spotted ranged from a minimum of about 20 ng for free fatty acids to a maximum of 600–700 ng for TG. Replicate sample analyses on separate plates yielded coefficients of variation of 5.9% and 2.3% for sterols (200 ng mean) and TG (300 ng mean), respectively.

The vertical depth profiles of sterol, TG, fatty alcohol and free fatty acid concentration are typical of many bulk property profiles in the upper 1000 meters of the ocean (Fig. 7A,B). These profiles reflect the primary production of particulate organic carbon (POC) in the photosynthetic zone in surface waters and a rapid net loss of POC with depth due to zooplankton grazing and oxidation. However, the wax ester profile (Fig. 7B) does not follow this trend and indicates that a secondary process controls the production and destruction of this compound class in the water column.

A large change is seen in the percent contribution of the individual lipid classes with depth (Fig. 8A,B). Fatty alcohols and free fatty acids both compose less than 5% of the total nonpolar lipids at all depths while sterols, TG, wax esters show large variations. TG (Fig. 8B) decrease

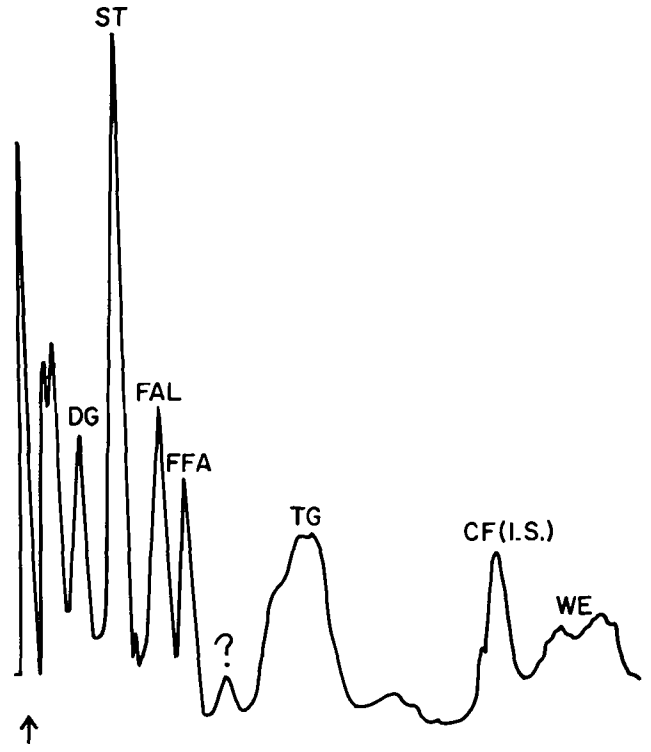

FIG. 6. Chromatogram of nonpolar lipid classes in a sample of oceanic particulate material from 450 m in the Slope Water in June 1982. Development was by Method I. The arrow indicates the preadsorbent zone/silica gel interface.

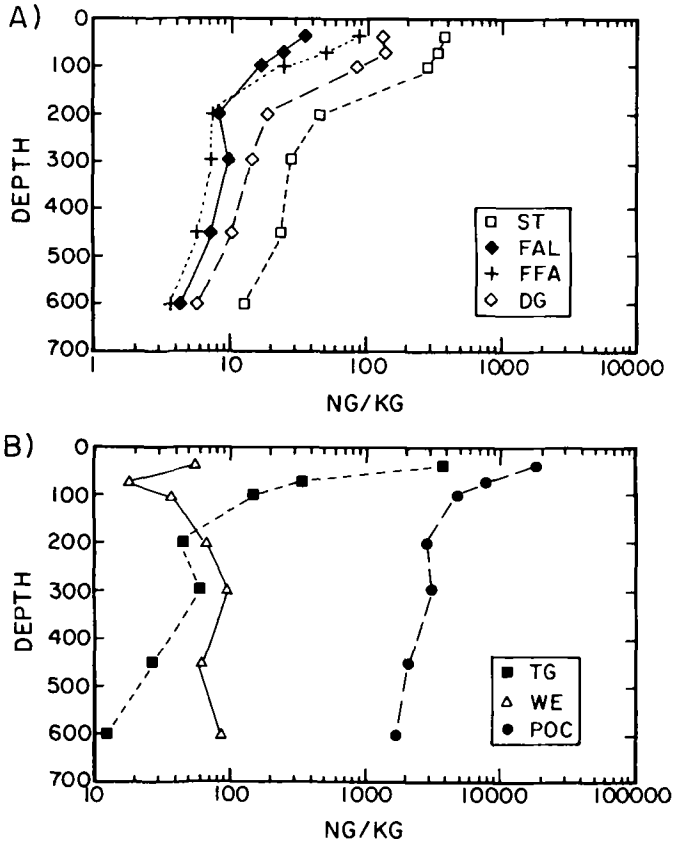

FIG. 7. Nonpolar lipid class concentrations in <53 μm particulate material in the Slope Water in June 1982. Concentration of total particulate organic carbon (POC) is given for comparison.

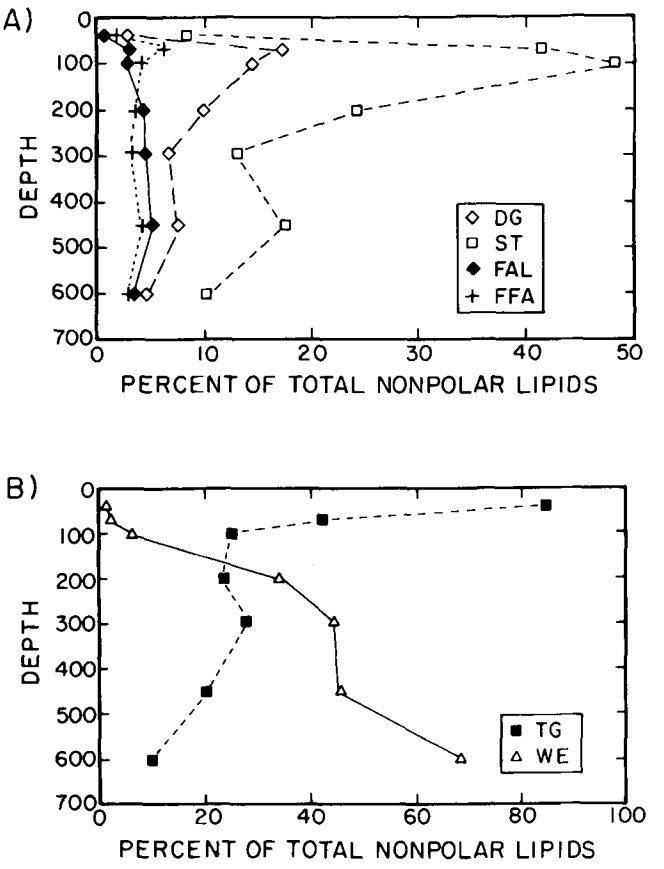

FIG. 8. Relative percentages of nonpolar lipid classes in <53 μ particulate material in the Slope Water in June 1982.

from over 80% of the total nonpolar lipids at the surface to only 10% in deep water. In contrast, wax esters show the reverse trend and increase from only a few percent of the total nonpolar lipid at the surface to 70% at 600 meters. This change in the dominant particulate lipid class with depth mirrors the general change with depth in the distributions of TG- and wax ester-producing organisms (9,45). Phytoplankton, which dominate the surface waters, synthesize almost exclusively TG. Zooplankton species may synthesize either TG or wax esters, but the major wax ester-synthesizing zooplankton in this temperate region are predominately meso- and bathypelagic. Thus, the increase with depth in the wax ester/TG ratio of the small particle pool most likely reflects changes in relative production of these two compounds by deep-living zooplankton. These results indicate that secondary production of POC by deep water zooplankton significantly alters the suspended particle pool in the water column.

Comparison of HPTLC with other methods. Quantitative HPTLC has several advantages over other methods used in lipid class analyses. The method presented here enables resolution and quantification of all the complex lipid classes in a crude lipid extract when the appropriate standards are run simultaneously on the same plate. Optimal amounts of lipid classes for highest resolution and accuracy in quantification range from 10 to 500 ng, which enables both major and trace lipid class concentrations to be determined from a single run. In addition, the lipid

specificity of the derivatization reaction in HPTLC minimizes interferences from pigments and other nonlipid compounds present in crude extract. In contrast, the Iatroscan/Chromarod method requires multiple developments and scans for resolution and quantification of all nonpolar lipid classes and requires extensive calibration of the flame ionization detector (FID) response to accurately quantify less than 1 μg of lipid classes.

The chromatographic methods used in this study are very simple and require no specialized equipment. The preadsorbent HPTLC plate eliminates many problems in HPTLC associated with applying very small volumes of crude extracts directly onto the silica gel surface. We have found that a simple, one-step application of dilute crude extracts with a Teflon-tipped pipette and development in standard chambers adequately resolves complex lipid classes. More sophisticated equipment and techniques will increase resolution but also increase cost and effort. Sensitivity in this study primarily was limited not by compound resolution but by the HPTLC plate. Further advances in HPTLC plate technology should continue to improve resolution and reduce the baseline problems associated with the plate surface.

The HPTLC method is rapid and requires no extensive calibration, so over 50 analyses easily can be made in one day. Run-to-run variability is less of a problem with HPTLC than with other methods because standards and samples are run simultaneously. In addition, the static nondestructive detection method allows samples to be scanned many times and in different modes to optimize quantification. In contrast, chromatographic conditions for Iatroscan/Chromarod analyses must be rigidly controlled for accurate results and detection by FID is destructive. Although the scanning densitometer for HPTLC is not inexpensive, it is not dedicated to a single analytical procedure. Therefore, HPTLC may be very cost effective because many diverse HPTLC methodologies may share the same instrument for detection.

We found quantitative HPTLC to be a rapid, simple and very sensitive technique for the analysis of complex lipid class composition in our samples of oceanic particulate material. The method shows much promise for other environmental sample analyses. In addition, the resolution and high sensitivity of the method makes it ideally suited for quantification of lipid classes before preparative scale separations by column chromatography, HPLC or TLC, and for purity checks of lipid fractions.

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REFERENCES

1. Wakeham, S.G. (1982) *Geochim. Cosmochim. Acta 46,* 2239-2257.
2. Wakeham, S.G., Lee, C., Farrington, J.W., and Gagosian, R.B. (1984) *Deep-Sea Res. 31,* 509-528.
3. Wakeham, S.G. (1985) *Mar. Chem. 17,* 213-235.
4. Saliot, A., Goutx, M., Fevrier, A., Tusseau, D., and Andrie, C. (1982) *Mar. Chem. 11,* 257-278.
5. Volkmann, J.K., Johns, R.B., Gillan, F.T., Perry, G.J., and Bavor, H.J. Jr. (1980) *Geochim. Cosmochim. Acta 44,* 1133-1143.
6. Sargent, J.R., Hopkins, C.C.E., Seiring, J.V., and Youngson, A. (1983) *Mar. Biol. 76,* 87-94.
7. Van Vleet, E.S. and Quinn, J.G. (1979) *Geochim. Cosmochim. Acta 43,* 289-303.
8. Fricke, H., Gerken, G., Schrieber, W., and Oehlenschlager, J. (1984) *Lipids 19,* 821-827.
9. Lee, R.F., and Hirota, J. (1973) *Limnol. Ocean. 18,* 227-239.
10. Dugan, L.L., Demediuk, P., Pendley, C.E. II, and Horrocks, L.A. (1986) *J. Chromatogr. 378,* 317-327.
11. Lapin, B.P., Pisareva, N.A., Rubtsova, T.E., and Jakevich, M.L. (1986) *J. Chromatogr. 365,* 229-235.
12. Kattner, G., Gerken, G., and Hemmer, K.D. (1983) *Mar. Chem. 14,* 163-173.
13. Pollero, R.J., Brenner, R.R., and Gros, E.G. (1981) *Lipids 16,* 109-113.
14. Sargent, J.R., and Falk-Petersen, S. (1981) *Mar. Biol. 62,* 131-137.
15. Delmas, R.P., Parrish, C.C., and Ackman, R.G. (1984) *Anal. Chem. 56,* 1272-1277.
16. Farnworth, E.R., Thompson, B.K., and Kramer, J.K.G. (1982) *J. Chromatogr. 240,* 463-474.
17. Hakanson, J.L. (1984) *Limnol. Oceanogr. 29,* 794-804.
18. Parrish, C.C., and Ackman, R.G. (1983) *J. Chromatogr. 262,* 103-112.
19. Sasaki, G.C., and Capuzzo, J.M. (1984) *Comp. Biochem. Physiol. 78B,* 525-531.
20. Sebedio, J.L., Astorg, P.O., Septier, C., and Grandgirard, A. (1987) *J. Chromatogr. 405,* 371-378.
21. Tatara, T., Fujii, T., Kawase, T., and Minagawa, M. (1983) *Lipids 18,* 732-736.
22. Parrish, C.C., and Ackman, R.G. (1985) *Lipids 20,* 521-530.
23. Terabayashi, T., Ogawa, T., Kawanishi, Y., Tanaka, M., Takase, K., and Ishii, J. (1986) *J. Chromatogr. 367,* 280-285.
24. Fenimore, D.C., and Davis, C.M. (1981) *Anal. Chem. 53,* 252A-259A.
25. Issaq, H. (1987) *Chromatography 2,* 37-41.
26. Halpaap, P. (1978) *Merck-Kontakte 1,* 32.
27. Yao, J.K., and Rastetter, G.M. (1985) *Anal. Biochem. 150,* 111-116.
28. Larsen, H.F., and Trostmann, A.F. (1981) *J. Chromatogr. 226,* 484-487.
29. Segura, R., and Gotto, A.M. (1974) *J. Chromatogr. 99,* 643-657.
30. Segura, R., and Navarro, X. (1981) *J. Chromatogr. 217,* 329-340.
31. Zhou, L., Shanfield, H., Wang, F.-S., and Zlatkis, A. (1981) *J. Chromatogr. 217,* 341-348.
32. Blass, K.G., and Ho, C.S. (1081) *J. Chromatogr. 208,* 170-173.
33. Kupke, I.R., and Zeugner, S. (1978) *J. Chromatogr. 146,* 261-271.
34. Kraus, U., Wurthe, J., and Rufer, R. (1987) *J. Chromatogr. 413,* 257-263.
35. Schmitz, G., Assmann, G., and Bowyer, D.E. (1984) *J. Chromatogr. 307,* 65-79.
36. Zhou, L., Shanfield, H., and Zlatkis, A. (1982) *J. Chromatogr. 239,* 259-264.
37. Bishop, J.K.B., and Edmond, J.M. (1976) *J. Mar. Res. 34,* 181-198.
38. Bishop, J.K.B., Schupack, D., Sherrell, R.M., and Conte, M.H. (1985) *Adv. in Chem. Series 209,* 155-175.
39 Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
40. Kaluzny, M.A., Duncan, L.A., Merritt, M.V., and Epps, D.E. (1985) *J. Lipid Res. 20,* 135-140.
41. Lillington, J.M., Trafford, D.J.H., and Makin, H.L.J. (1981) *Clin. Chem. Acta 111,* 91-98.
42. Macala, L.J., Yu, R.K., and Ando, S. (1983) *J. Lipid Res. 24,* 1243-1250.
43. Nutter, L.J., and Privett, O.S. (1968) *J. Chromatogr. 35,* 519-525.
44. Pollero, R.J. (1983) *Lipids 18,* 12-17.
45. Sargent, J.R. (1976) in *Biochemical and Biophysical Perspectives in Marine Biology* (Malins, D.C., and Sargent, J.R., eds.) Vol. 3, pp. 149-212, Academic Press, New York.

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Influence of Diet on (n-3) and (n-6) Fatty Acids in Monkey Erythrocytes

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Cynomolgus monkeys were fed oils high in linoleic acid or with half of the linoleic acid replaced by either (n-3) linolenic acid or marine fatty acids. When the diet contained similar quantities of linoleic and (n-3) linolenic acid, erythrocyte fatty acids maintained a ratio of (n-6) to (n-3) fatty acids of approximately 2:4. Fatty acids from menhaden oil enhanced the incorporation of eicosapentaenoic and docosahexaenoic acids into the monkey erythrocytes, the composition of which was not altered by additional α-tocopherol.
Lipids 23, 501-503 (1988).

Erythrocytes are suitable cells for detecting the prolonged intake of (n-3) fatty acids in rats (1) and the best available retrospective index of the types of fat ingested by humans (2). When both linoleic acid and (n-3) linolenic acid are provided, desaturases favor (n-3) metabolism (3), but transacylases preferably catalyze the incorporation of arachidonic acid (4).

This investigation on erythrocytes of the cynomolgus monkey was undertaken to determine the effect of equal dietary levels of linoleic acid and different (n-3) fatty acids. These were provided by either α-linolenic acid from linseed oil or C_{20} and C_{22} polyunsaturated fatty acids from menhaden oil. The fish oil also was fed with additional tocopherol to protect the polyunsaturated fatty acids.

MATERIAL AND METHODS

Twenty male and 20 female cynomolgus monkeys *(Macaca fascicularis)* of five to nine years of age previously had been used for testing poliomyelitis vaccine at Connaught Laboratories, Toronto, Ontario, and then for testing zinc supplements in the Department of National Health and Welfare, Ottawa. They were given a four-month rest period, taught to eat dough balls containing purified diet ingredients and orange flavoring, and distributed among four test groups on the basis of body weight and sex.

The basal diet contained in % by weight: sucrose, 26.45; corn starch, 21.07; casein, 21.43; alphacel, 10.00; Ausman-Hayes mineral mix, 4.75; Newberne-Hayes vitamin mix (5), 0.50; D,L-methionine 0.50; fat, 15.00. The Ausman-Hayes mineral mix contained, in g/kg, potassium phosphate, dibasic, K_2HPO_4, 327.908; calcium carbonate, $CaCO_3$, 290.485; sodium chloride, NaCl, 162.366; magnesium sulfate, $MgSO_4$-$7H_2O$, 98.732; calcium phosphate, dibasic, $CaHPO_4$-$2H_2O$, 72.597; magnesium oxide, MgO, 32.040; ferric citrate, (16.7 % Fe), 13.297; manganese sulphate, $MnSO_4$-H_2O, 1.221; zinc chloride, $ZnCl_2$, 0.915; cupric sulphate, $CuSO_4$-$5H_2O$, 0.290; potassium iodide, KI, 0.077; chromium acetate, $Cr(C_2H_3O_2)_3$, 0.044; sodium fluoride, NaF, 0.023; sodium selenite, Na_2SeO_3, 0.004. A 2.5% agar solution was mixed with the basal diet in a 2:5 ratio to produce dough and reduce surface exposed to air. The diet was made each week, divided into daily portions and stored at 4 C until fed.

The fat in diet 1 was 63.75% lard and 36.25% corn oil; diet 2, 66.0% lard and 34.0% linseed oil; diets 3 and 4, 15.6% lard, 16.1% corn oil and 68.3% menhaden oil. The fatty acid composition of each diet is given in Table 1. Diet 4 was supplemented to contain 213 μg α-tocopherol/g diet, whereas diet 3 contained 49 μg/g.

Blood was drawn into a vacutainer tube containing sodium citrate from animals immobilized with 5 mg/kg

TABLE 1

Fatty Acid Composition of Diets[a]

Fatty acid	Diet 1	Diet 2	Diet 3	Diet 4
12:0	0.7	1.1	2.1	0.4
14:0	2.1	2.3	12.9	12.9
14:1			0.9	0.7
14:1 isomers			0.2	0.5
16:0	28.6	27.5	25.1	25.8
16:1(n-7)	2.5	2.6	10.5	10.8
16:1 isomers			0.5	0.3
17:0	0.3	0.3	0.1	0.2
17:1	0.3	0.3	1.4	1.5
17:1 isomers			0.5	0.5
18:0	7.7	8.4	3.6	3.7
18:1(n-9)	32.1	32.4	16.4	16.9
18:1 isomers	0.2	0.2	1.5	1.5
18:2(n-6)	24.7	11.8	10.6	10.8
18:2 isomers			1.6	1.5
18:3(n-3)	0.4	13.0	0.9	0.8
18:3(n-6)			0.1	0.1
18:4(n-3)			1.8	1.9
19:0			0.1	0.1
20:0	0.3	0.1		
20:1(n-9)	0.2		0.4	0.5
20:1 isomers			0.1	0.2
20:2(n-6)	0.1		0.2	0.3
20:4(n-3)			0.3	0.4
20:5(n-3)			4.7	4.6
22:0			0.2	
22:1(n-11)			0.3	
22:1 isomers				0.5
22:5(n-3)			0.4	0.2
22:6(n-3)			2.8	2.4
(n-6)	24.8	11.8	10.8	11.2
(n-3)	0.4	13.0	10.9	10.2
(Monounsaturates)	35.2	35.5	32.8	35.5
(Saturates)	39.7	39.7	43.9	43.1
(n-6)/(n-3)	64.0	0.9	1.0	1.1

[a]Fat was extracted from the diets three times during the experiment; the fatty acids are expressed as area % of chromatograms.

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ketamine hydrochloride (Rogarsetic, Bristol Laboratories, Syracuse, NY). The hematological profile was obtained on a Coulter counter (Model S. Plus IV, Mississauga, Ontario). The blood was fractionated (1) and a weighed sample of purified erythrocytes extracted (6) and stored at 4 C. A portion of this was methylated (7) for gas chromatography on a fused silica column, Supelcowax 10, 30 m × 0.32 mm i.d., film of 0.25 μm, in a Varian Vista 6000 instrument with hydrogen as the carrier gas. The oven temperature was 130 C for 12 min, then increased 2 degrees per minute to 180 C for 18 min. The injector was at 250 C, and the detector was at 275 C. Calibration was achieved with the methyl ester of nonadecanoic acid as the internal standard. Results were expressed as μg fatty acid per 10^6 erythrocytes and subjected to statistical analysis of variance and Duncan's range test (8).

RESULTS AND DISCUSSION

The erythrocyte (n-6) and (n-3) fatty acids, determined on each monkey at intervals (Fig. 1), are given for the final period in Table 2. Group 1, fed a high level of linoleic acid and a low level of (n-3) linolenic acid, showed gradually increased concentrations of (n-6) fatty acid in erythrocytes with time, while group 2 fed approximately equal amounts of linoleic and (n-3) linolenic acid maintained a relatively constant proportion of total (n-6) to (n-3) fatty acids throughout the experimental feeding period. In contrast, the monkeys fed menhaden oil as a source of (n-3) fatty acids, groups 3 and 4, showed elevated 20:5 and 22:6 fatty acids as replacements for (n-6) fatty acids. Additional tocopherol had no effect on the composition of the erythrocyte fatty acids.

The diets had an equal distribution of saturated, monounsaturated and polyunsaturated fatty acids, the last contributing the essential fatty acids for the 2-position of glycerophosphatides, which with cholesterol compose most of the erythrocyte lipid (9). When the diet supplied similar amounts of linoleic acid and (n-3) linolenic acid, the ratio of the total (n-6) to (n-3) fatty acids remained at about 2:4. This indicated a close regulation for the conversion to long chain fatty acids and their incorporation into the cell. The long chain (n-3) fatty acid from fish oil, increased progressively in the erythrocyte. The elimination of the need for desaturation and elongation of substrates apparently resulted in less metabolic regulation of erythrocyte fatty acid composition.

The enhanced incorporation of the marine-type compared with the plant-type (n-3) fatty acids has been observed in human platelets (6,10,11) and rat tissues, notably the heart (1,12). Erythrocytes of the rabbit maintained a constant level of arachidonic acid when either (n-3) or (n-6) fatty acids was fed (13), but those of the marmoset monkey exhibited differences in arachidonic acid with dietary sheep perirenal fat or sunflowerseed oil, both low in (n-3) fatty acids (14). The respective n-6/n-3 ratios were 3.0 and 6.4, both of which were higher than those observed in the present experiment involving substantial

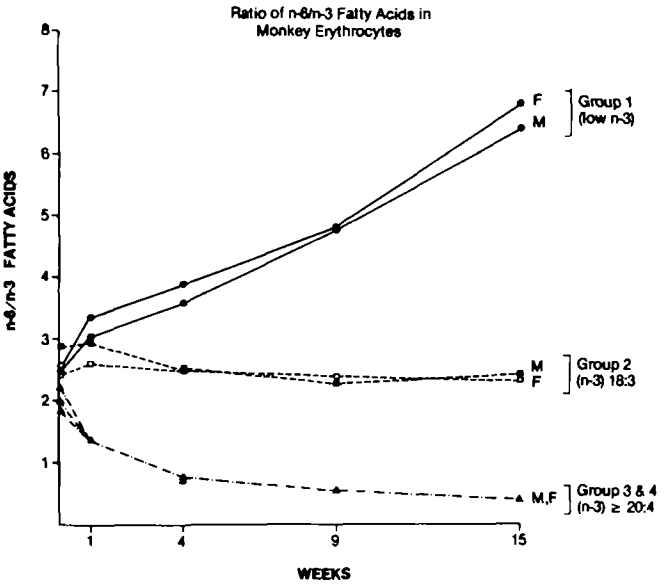

FIG. 1. The ratio of total (n-6) to (n-3) fatty acids of erythrocytes from monkeys fed different dietary fats during 15 wk.

TABLE 2

Polyunsaturated Fatty Acids in Monkey Erythrocytes After 15 Weeks of Test Diets (μg/10^4 Cells)

	Group 1		Group 2		Group 3		Group 4	
Fatty acids	Male	Female	Male	Female	Male	Female	Male	Female
(n-6) 18:2	2.31±0.14*,a	1.87±0.11a,b,c	1.76±0.15b	1.61±0.11b	0.76±0.06c	0.64±0.04c	0.79±0.09c	0.69±0.11c
20:4	1.82±0.11a	1.67±0.09a,c	1.40±0.15a,c	1.40±0.13a,c	0.48±0.04b,c	0.44±0.05b,c	0.48±0.05b,c	0.41±0.05b,c
22:4	0.29±0.02	0.26±0.03	0.06±0.01	0.08±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01
22:5	0.06±0.01	0.06±0.01	0.03±0.01	0.03±0.01	0.01±0.01	0.01±0.01	0.02±0.01	0.01±0.01
Total	4.54±0.17a	3.94±0.21a,b,c	3.32±0.28b	3.19±0.25b	1.26±0.09c	1.10±0.9c	1.33±0.11c	1.14±0.16c
(n-3) 18:3	0.00±0.00b	0.00±0.00b	0.21±0.02a	0.18±0.02a	0.00±0.00b	0.00±0.00b	0.05±0.03b	0.00±0.00b
20:4	0.00±0.00	0.00±0.00	0.03±0.01	0.04±0.01	0.03±0.01	0.02±0.01	0.03±0.01	0.03±0.01
20:5	0.04±0.01b	0.03±0.01b	0.31±0.03b	0.28±0.01b	1.43±0.09a	1.39±0.12a	1.55±0.17a	1.42±0.16a
22:5	0.20±0.02	0.16±0.02	0.39±0.02	0.45±0.04	0.32±0.01	0.30±0.02	0.35±0.04	0.37±0.05
22:6	0.48±0.02b	0.42±0.05b	0.42±0.04b	0.43±0.04b	1.23±0.10a	1.30±0.12a	1.27±0.14a	1.29±0.17a
Total	0.73±0.05b	0.61±0.08b	1.37±0.10b	1.37±0.07b	3.01±0.18a	3.01±0.26a	3.25±0.30a	3.11±0.37a

*Mean ± standard error of the mean. Means in a horizontal line not sharing the same superscript are statistically different at p = 0.01.

quantities of dietary (n-3) fatty acids. A supply of preformed fatty acid substrates permitted an enhanced concentration of them in membrane lipids. This may be advantageous in some situations but may circumvent some metabolic control steps.

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REFERENCES

1. Roshanai, F., and Sanders, T.A.B. (1985) *Ann. Nutr. Metab. 29,* 189-196.
2. Simpson, H.C.R., Barker, K., Carter, K.D., Cassels, E., and Mann, J.I. (1982) *Br. Med. J. 285,* 683-684.
3. Brenner, R.R., and Peluffo, R. (1966) *J. Biol. Chem. 241,* 5213-5219.
4. Iritani, N., and Fujikawa, S. (1982) *J. Nutr. Sci. Vitaminol. 28,* 621-629.
5. Alderson, L.M., Hayes, K.C., and Nicolosi, R.J. (1986) *Arteriosclerosis 6,* 465-474.
6. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
7. Beare-Rogers, J.L., Gray, L., Nera, E.A., and Levin, O.L. (1979) *Nutr. Metab. 23,* 335-346.
8. Kirk, R.E. (1968) in *Experimental Design: Procedures for the Behavioral Sciences,* pp. 93-94, Brooks-Coles Publishing Co., Pacific Grove, CA.
9. Schwartz, R.S., Chiu, D.T.-Y., and Lubin, B. (1985) *Curr. Topics Haematol. 5,* 63-112.
10. Sanders, T.A.B., Vickers, M., and Haines, A.P. (1981) *Clin. Sci. 61,* 317-324.
11. Galloway, J.H., Cartwright, I.J., Woodcock, B.E., Greave, M., Graham, R.G., Russell, G., and Preston, F.E. (1985) *Clin. Sci. 68,* 449-454.
12. Holmer, G., and Beare-Rogers, J.L. (1985) *Nutr. Res. 5,* 1011-1014.
13. Housley, G., Born, G.V.R., Conroy, D.M., Berlin, J., and Smith, A.D. (1986) *Proc. R. Soc. London B 227,* 43-51.
14. McMurchie, E.J., Gibson, R.A., Charnock, J.S., and McIntosh, G.H. (1986) *Lipids 21,* 315-323.

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Mammary Lipoprotein Lipase in Plasma of Cows After Parturition or Prolactin Infusion

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Plasma lipase activity from the mammary vein and a tail blood vessel was measured in periparturient Holstein cows treated in one of three ways: control, CB154 (2-Br-α-ergocryptin) or CB154 plus prolactin. CB154 administration decreased basal serum prolactin concentration by 80% and blocked the normal parturient increase of serum prolactin. In CB154 plus prolactin-treated cows, prolactin was infused continuously for six days starting five and eight days prepartum. Plasma lipase activity was not detectable up to 26 hr prepartum in control and CB154-treated cows or before the start of prolactin infusion in CB154 plus prolactin-treated cows. After two hr prepartum, plasma lipase activity was detected in all treatments. In CB154 plus prolactin-treated cows, plasma lipase activity was detected in the presence of high concentrations of serum progesterone four days after the start of prolactin infusion and at least two days before parturition. Plasma lipase activity was four times greater in the mammary vein than in the tail vessel at sampling times at which activity was detected in both vessels. We propose the difference between plasma lipase activity from the mammary vein and tail vessel is due to release of lipoprotein lipase from the mammary gland into blood, and this activity can be induced prepartum by prolactin or at parturition even if the parturient increase in prolactin is suppressed.

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Approximately 40% of bovine milk fat is derived from blood triglycerides (1) that have been hydrolyzed by lipoprotein lipase (LPL) at the luminal surface of the mammary gland capillary endothelium (2). LPL at the capillary endothelium usually is measured in plasma after heparin injection. However, lipase activity in plasma without prior injection of heparin has been measured in the lactating goat (3) and cow (4). This plasma lipase activity can be attributed in part to mammary LPL because the concentration of plasma lipase activity is greater in the mammary vein than in the mammary artery (3,4) and is inhibited by NaCl (3). This plasma lipase activity from the mammary vein of lactating cows has been reported by Shirley (4) to be 0.5% of that found after heparin injection.

LPL activity in mammary tissue increases markedly just before parturition and remains high throughout lactation in the guinea pig (5,6), rat (7) and cow (8). Prolactin increases mammary LPL activity in the pseudopregnant rabbit (9) and reestablishes mammary LPL activity after hypophysectomy in the lactating rat (10). But when prolactin secretion is inhibited by CB154 (2-Br-α-ergocryptin) in 20-day pregnant rats, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) increases mammary LPL activity while decreasing serum progesterone concentration (11). $PGF_{2\alpha}$ may be acting by reducing serum progesterone concentration because when progesterone and $PGF_{2\alpha}$ are administered together, mammary LPL activity does not increase.

The purpose of this study was to investigate plasma lipase activity in the periparturient cow and to show that most of this activity is due to mammary LPL released into the blood. The independent effects of prolactin and parturition on plasma lipase activity also are described. Other aspects of this study are reported (12,13).

MATERIALS AND METHODS

Treatments. Details of the experimental design and care of the animals are reported (12). The three treatment groups were control (n = 3), CB154 (n = 3) and CB154 plus prolactin (n = 2). For CB154 and CB154-plus-prolactin-treated cows, treatment with CB154 (Sandoz Inc., E. Hanover, NJ) commenced 12 days before expected parturition and continued through 10 days postpartum. CB154 (11 mg/100 kg BW) was injected subcutaneously either every four days, every two days or daily prepartum. All schedules of CB154 injection reduced concentration of prolactin in serum similarly (12). After parturition, CB154 and CB154 plus prolactin-treated cows were injected every two days.

Cows assigned to treatment with CB154 plus prolactin were infused with 6.0, 8.0, 9.8, 22.4 and 20.8 mg prolactin/hr (NIH-B4; NIH, Bethesda, MD) for 24 hr each, starting five days before the expected day of parturition. Infusions started at 2400 hr and rates were changed at 24-hr intervals (12).

Blood sampling and hormone assays. Blood for lipase assay was collected once every three days between 0900 and 1100 hr starting two wk before estimated calving date. Blood was obtained from the subcutaneous abdominal vein (Mammary vein) and the coccygeal vein or artery (tail vessel) in tubes containing potassium oxalate (2 mg/ml blood). Plasma was removed after centrifugation at 4 C and stored at −50 C until assayed 12 to 18 months after samples were taken. A standard stored with the samples showed no decrease in activity over the same period.

Blood samples for prolactin and progesterone determinations were taken twice daily (0700 and 1900 hr) from an indwelling jugular cannula one hr before feeding and milking. Hormones were quantified as described (12).

Lipase assay. Tri-[9,10(n)-^{3}H]oleoylglycerol, [9,10(n)-^{3}H]oleic acid and ACS scintillation fluid were obtained from Amersham (Arlington Heights, IL). Labeled triacylglycerol was purified by Florisil column chromatography (14) and was used within two months of chromatography. Unlabeled trioleoylglycerol, bovine serum albumin, Trizma base and Triton X-100 (acetyl phenoxy polyethoxyethanol) were obtained from Sigma Chemical Co. (St. Louis, MO). All other substances were reagent-grade, and all solvents were distilled before use.

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Abbreviations: HTGL, hepatic triglyceride lipase; LPL, lipoprotein lipase.

The substrate mixture in a polyethylene 20 ml scintillation vial contained 150 μmol trioleoylglycerol; 70 μCi tri-[9,10(n)-^{3}H]oleoylglycerol; 2.5 ml heat-inactivated bovine serum; 8.1 ml 8% bovine serum albumin, 0.15 M NaCl, 0.24 M Tris-HCl (pH 8.6); and 0.9 ml 1% Triton X-100. The vial was placed in ice and the substrate mixture sonicated three times for one min with one-min pauses with a sonifier cell disruptor (Heat Systems-Ultrasonic, Plainview, NY, Model W185D) using a micro tip and a setting of 75 W.

Culture tubes (12 × 75 mm) for the lipase assay contained 0, 40 or 60 μl of sample plasma and 40 μl 3% bovine serum albumin with either 0.15 or 4.4 M NaCl. Enough heat-inactivated bovine serum was added to bring the volume to 100 μl. The incubation was started by adding 100 μl substrate mixture (final trioleoylglycerol concentration, 6.5 mM) and incubating in a shaking water bath at 37 C. After one hr, the assay was stopped and released [^{3}H]oleic acid separated from the acylglycerols with 1.65 ml methanol/chloroform/heptane (1.41:1.25:1, v/v/v) and 0.45 ml of alkaline buffer (58 mM sodium borate, 41 mM potassium carbonate titrated to pH 10.4 with 5 M potassium hydroxide) (15). After centrifugation at 20 C, 0.5 ml of the upper phase was transferred to scintillation vials with 10 ml of ACS scintillation fluid. Recovery of oleic acid in the 0.5 ml transferred was 25% as determined by replacing labeled trioleoglycerol with labeled oleic acid in the substrate mixture. Activity in the tubes containing 60 μl heat-inactivated bovine serum was subtracted from sample tubes to correct for background. Lipase activity for a sample was determined in duplicate at 40 and 60 μl of plasma and expressed as nmol fatty acid (FA)/hr/ml of plasma. Minimum detectable activity was 60 nmol FA/hr/ml plasma. Lipase activity was linear with time and plasma concentration.

Statistical methods. Plasma lipase activity for samples taken between two hr prepartum and 114 hr postpartum were analyzed by split plot analysis of variance (16). Cows were sampled once in period 1 from two hr prepartum to 52 hr postpartum and again in period 2 from 70 hr to 114 hr postpartum (Fig. 1). Samples taken in these two periods with plasma lipase activity below the detectable limit were set to zero and included in the analysis. Plasma lipase activity for samples from CB154 plus prolactin-treated cows taken after the start of prolactin infusion and before parturition were analyzed using Student's t-test.

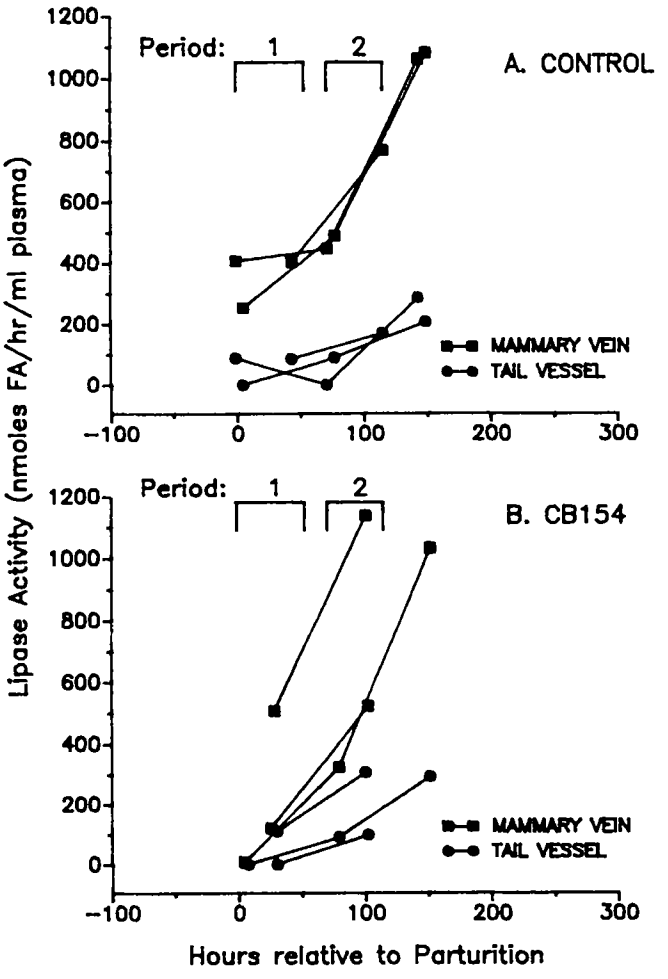

FIG. 1. Plasma lipase activity from the mammary vein and tail vessel for individual cows. (A) control cows; (B) CB154-treated cows. Cows were sampled once in each period.

RESULTS

Lipase activity. Lipase activity was not detectable in any plasma samples taken up to 26 hr prepartum in control and CB154 treated cows (sampling range is 1 to 14 days prepartum). Plasma lipase activity was detected in all but one of the mammary vein samples and most tail vessel samples after parturition in control, CB154 and CB154 plus prolactin-treated cows (Figs. 1 and 2). Between 2 hr prepartum and 120 hr postpartum, there were no significant differences among treatments or a significant treatment by period interaction. Mammary vein plasma lipase activity increased from period 1 to 2 ($P < 0.01$, Figs. 1 and 2).

In the two CB154 plus prolactin-treated cows, plasma lipase activity was detected in mammary vein plasma 82 and 106 hr after the start of prolactin infusion (Fig. 2). This was 92 and 43 hr before parturition, respectively. Plasma lipase activity was detected in all mammary vein and tail vessel samples 106 hr after the start of infusion.

In all treatments, lipase activity detected in mammary vein plasma was greater than tail vessel plasma lipase activity. Plasma lipase activity from the tail vessel was 23% that of the mammary vein plasma for observations in which activity was detected in both samples (range 18%-29%, n = 17).

Hormones. Results of hormone data have been reported (12). In control cows, serum prolactin averaged 29 ng/ml six to two days prepartum, reached an average peak of 194 ng/ml at parturition and declined to an average 28 ng/ml 2-10 days postpartum. Treatment with CB154 reduced serum prolactin concentration approximately 80% to 6 ng/ml. Serum prolactin concentration for CB154 plus prolactin-treated cows was similar to that for CB154 treated cows except during the time of prolactin infusion when serum prolactin concentration reached a peak of 350 ng/ml on day 5 of the infusion and then declined to its preinfusion level within six hr of the end of infusion. Treatment with CB154 and CB154 plus prolactin had no effect on the concentration of serum progesterone five

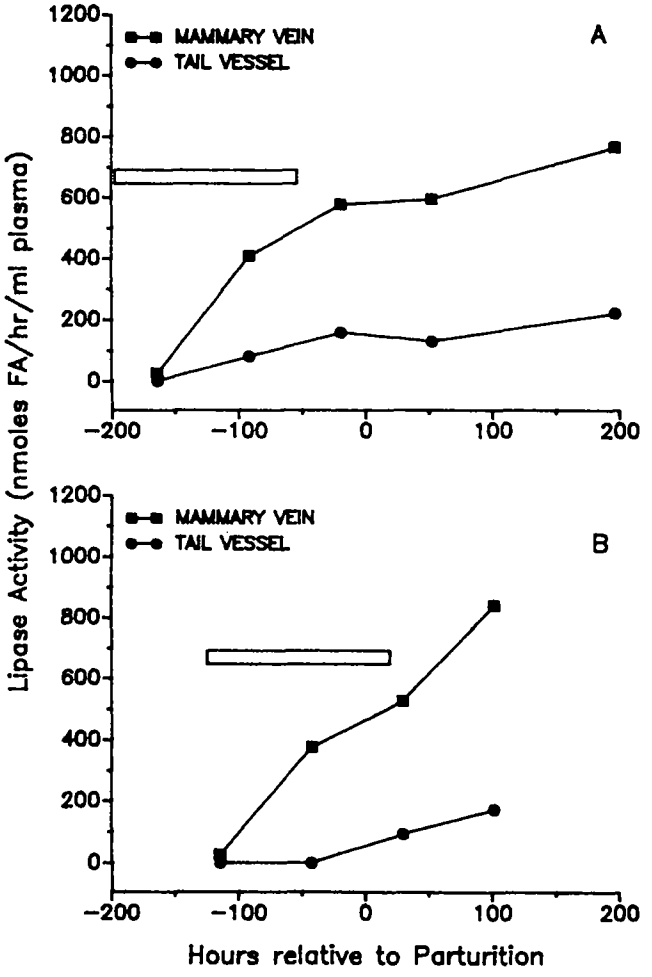

FIG. 2. Plasma lipase activity from the mammary vein and tail vessel during periparturient period for two cows treated with CB154 plus prolactin. (A) cow 1; (B) cow 2. Bars indicate time of prolactin infusion.

days prepartum to three days postpartum when compared with control. Between day 2 prepartum and day 2 postpartum, serum progesterone declined from an average of 6 ng/ml to less than 1 ng/ml.

DISCUSSION

Mammary tissue LPL activity is virtually absent during most of pregnancy in non-lactating animals and increases rapidly 24 to 48 hr before parturition in the guinea pig (5,6), rat (7) and cow (8). In this study, plasma lipase activity in control and CB154-treated cows followed a similar pattern (Fig. 1). The higher plasma lipase activity in the mammary vein relative to the tail vessel suggests that the difference in lipase activity between the two vessels is due to LPL released from the mammary gland. This agrees with the findings of Barry et al. (3) in the lactating goat in which the plasma lipase activity was completely inhibited by 0.8 M NaCl—a characteristic of LPL. But, in both tail vessel and mammary vein plasma from lactating cows, we found only an 80% inhibition of plasma lipase activity with 1 M NaCl. This is similar, though, to the 70% reduction in activity we found with plasma LPL purified on heparin-sepharose and assayed without activator (17). The increase in tail vessel plasma lipase activity at the same time as that in the mammary vein suggests that most of the activity in tail vessel plasma also is due to mammary LPL that has not yet been cleared from the blood. This conclusion is supported by the reports showing that adipose tissue LPL declines with onset of lactation (8) and that the other major plasma lipase, hepatic triglyceride lipase (HTGL), is either absent (18) or very low (17,19) in post-heparin plasma of cows.

Prolactin has been shown to increase mammary LPL activity in pseudopregnant rabbits (9) and to re-establish mammary LPL activity after hypophysectomy in lactating rats (10). In this study, infusion of prolactin into CB154 plus prolactin-treated cows was to mimic the normal increase in serum prolactin in the cow at parturition (20). Although plasma lipase activity was not detected until 82 hr after the start of infusion, it was measurable in both cows before the decline in serum progesterone, two days prepartum (Fig. 2). Spooner et al. (11), though, found no increase in mammary LPL activity when prolactin was given to 20-day pregnant rats with high concentrations of serum progesterone; but serum prolactin concentration was not measured and may not have been high enough to effect mammary LPL.

In CB154-treated cows, plasma lipase activity increased after parturition despite low concentrations of serum prolactin. This induction of plasma lipase activity may be caused by the decline in serum progesterone concentration in the presence of low serum prolactin concentration. In 20-day pregnant rats treated with CB154 and $PGF_{2\alpha}$, the serum concentrations of progesterone and prolactin decline and mammary LPL activity increases (11). Djiane and Durand (21) have shown that progesterone blocks the induction of prolactin receptors and prolactin stimulates the formation of its own receptors in the rabbit mammary gland. For the CB154-treated cows, the decline in serum progesterone concentration started two days prepartum and, although low, serum prolactin concentration was not completely suppressed by CB154.

After hydrolysis of serum triglycerides, the probable fate of LPL at the capillary endothelium is to be released into the circulation for catabolism at the liver (22,23). The increased hydrolysis of serum triglycerides by mammary LPL during lactation then would be the cause of the plasma lipase activity measured in this study. This is supported by Shirley (4), who observed that post-heparin plasma lipase activity increased eight-fold after parturition and most of this increase could be attributed to greater plasma lipase activity from the mammary vein. We conclude that the lipase activity in plasma from periparturient cows is mostly due to LPL from the mammary gland and that this activity can be induced prepartum by high serum concentrations of prolactin or at parturition with or without the parturient increase of serum prolactin.

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REFERENCES

1. Glascock, R.F., Welch, V.A., Bishop, C., Davies, T., Wright, E.W., and Noble, R.C. (1966) *Biochem. J. 98*, 149-156.
2. Moore, J.H., and Chistie, W.W. (1981) in *Lipid Metabolism in*

Ruminant Animals (Christie, W.W., ed.), pp. 227-277, Pergamon Press Ltd., Oxford, U.K.
3. Barry, J.M., Bartley, W., Linzell, J.L., and Robinson, D.S. (1963) *Biochem. J. 89,* 6-11.
4. Shirley, J.E. (1973) Regulatory Aspects of Triglyceride Uptake by Bovine Adipose and Mammary Tissues, Ph.D. Thesis, Michigan State University, East Lansing, MI.
5. McBirde, O.W., and Korn, E.D. (1963) *J. Lipid Res. 4,* 17-20.
6. Robinson, D.S. (1963) *J. Lipid Res. 4,* 21-23.
7. Hamosh, M., Clary, T.R., Chernick, S.S., and Scow, R.O. (1970) *Biochim. Biophys. Acta 210,* 473-482.
8. Shirley, J.E., Emery, R.S., Convey, E.M., and Oxender, W.D. (1973) *J. Dairy Sci. 56,* 569-574.
9. Falconer, I.R., and Fiddler, T.J. (1970) *Biochim. Biophys. Acta 218,* 508-514.
10. Zinder, O., Hamosh, M., Fleck, T.R.C., and Scow, R.O. (1974) *Am. J. Physiol. 226,* 744-748.
11. Spooner, P.M., Garrison, M.M., and Scow, R.O. (1977) *J. Clin. Invest. 60,* 702-708.
12. Akers, R.M., Bauman, D.E., Capuco, A.V., Goodman, G.T., and Tucker, H.A. (1981) *Endocrinology 109,* 23-30.
13. Akers, R.M., Bauman, D.E., Goodman, G.T., Capuco, A.V., and Tucker, H.A. (1981) *Endocrinology 109,* 31-40.
14. Carroll, K.K. (1961) *J. Lipid Res. 2,* 135-141.
15. Belfrage, P., and Vaughan, M. (1969) *J. Lipid Res. 10,* 341-344.
16. Gill, J.L. (1978) *Design and Analysis of Experiments in the Animal and Medical Sciences,* Vol. 2, pp. 203-214, The Iowa State University Press, Ames, IA.
17. Liesman, J., Emery, R.S., Gerloff, B., and Herdt, T. (1984) *Can. J. Anim. Sci. 64* (suppl.), 248-249.
18. Etienne, J., Noe, L., Rossignol, M., Dosne, A., and Debray, J. (1981) *Biochim. Biophys. Acta 663,* 516-523.
19. Cordle, S.R., Yeaman, S.J., and Clegg, R.A. (1983) *Biochim. Biophys. Acta 753,* 213-219.
20. Ingalls, W.G., Convey, E.M., and Hafs, H.D. (1973) *Proc. Soc. Exp. Biol. Med. 143,* 161-164.
21. Djiane, J., and Durand, P. (1977) *Nature 266,* 641-643.
22. Felts, J.M., Itakura, H., and Crane, R.T. (1975) *Biochem. Biophys. Res. Commun. 66,* 1467-1475.
23. Wallinder, L., Peterson, J., Olivecrona, T., and Bengtsson-Olivecrona, G. (1984) *Biochim. Biophys. Acta 795,* 513-524.

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Stimulation of Liver Growth and DNA Synthesis by Glucosylceramide

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The nature of the growth-stimulating effect of glucosylceramide was studied. Mice were injected intraperitoneally with emulsified glucosylceramide and conduritol B epoxide, an inhibitor of cerebroside glucosidase. Within one or two days, the liver grew 18–24%, as reported. Two enzymes involved in DNA synthesis also increased more than the weight. The total liver activity of thymidine kinase increased 46–73%, and the total activity of ornithine decarboxylase increased as much as 101%. It is suggested that elevated liver levels of glucocerebroside stimulate cell proliferation through a relatively direct mechanism.
Lipids 23, 508–510 (1988).

It is evident that the growth of liver and spleen in Gaucher patients, who lack a specific glucohydrolase (EC 3.2.1.45), is far more than can be accounted for by accommodation to the weight of stored glucosylceramide (GlcCer), the enzyme's substrate. The chemical nature of the enlarged organs and the mechanisms of the causational sequence still are obscure. In a study in which we injected mice intraperitoneally over an eight-day period with conduritol B epoxide (CBE), an inhibitor of GlcCer glucosidase, we found significant increases in liver and brain size (9% and 13%, respectively) (1). Presumably, the CBE caused an accumulation of the enzyme's substrate (2) and, therefore, induced a temporary model form of Gaucher disease. In a later study (3), we injected emulsified GlcCer once and found that much of the lipid reached the liver and that a rapid enlargement of the liver ensued, 10–37% within 25 hr. Co-injection of CBE with GlcCer enhanced the growth phenomenon. The enlargement was accompanied by an increase in total DNA, lipid and protein (as well as the glucosidase-activator proteins that are known to accumulate in Gaucher spleen); it was evident that the increase in liver size could not be attributed to edema. The detergent alone did not produce a change in liver weight. We have postulated that GlcCer can induce cell division and growth. The study reported here was directed at clarifying the mechanism by which this occurs.

METHODS AND MATERIALS

Materials. Most of the materials, the mice used and the method for selecting them for each experimental group have been described (3,4). The GlcCer, isolated from Gaucher spleen, was emulsified with 3/4 of its weight of Myrj 52, a low-toxicity nonionic detergent (polyoxyethylene stearate, ICI America Inc.), and injected i.p. into 16-day-old mice (3). Labeled thymidine was from ICN and labeled ornithine was from DuPont NEN.

Assays. Thymidine kinase was measured by following the phosphorylation of [^{3}H]thymidine, 182 cpm/nmol, in a tube containing Tris-Cl$^-$ pH 7.5, ethylenediamine tetraacetic acid (EDTA), mercaptoethanol, adenosine triphosphate (ATP), $MgCl_2$, NaF, bovine serum albumin, creatine phosphate and creatine kinase (5,6). The incubation with liver cytosol was performed for 30 min. The resultant thymidine phosphate was isolated with ion exchange paper.

Ornithine decarboxylase was measured by following the release of [^{14}C]O_2 from L-[1-^{14}C]ornithine (22.9 cpm/pmol) in EDTA, Tris-Cl$^-$ pH 7.4, DTT, pyridoxal phosphate (7, 8), and 2-methylornithine (in half of the tubes). Each assay tube contained the supernatant suspension from centrifugation of liver homogenates at 30,000 g for 25 min.

Protein and DNA were determined as described (3).

RESULTS

In one experiment, thymidine kinase specific activity was determined in the livers of mice injected with GlcCer/Myrj in saline, then (24 hr later) with CBE in saline. A day later, the mice were killed, and the livers were analyzed (Table 1). Control mice received two injections of saline alone. The previous observations, increases in protein and DNA that paralleled liver growth, were confirmed. In addition, a distinct rise in thymidine kinase total activity (46%) was seen.

In another experiment, GlcCer/Myrj and CBE were injected as a mixture once, and the animals were killed 24 or 48 hr later. Thymidine kinase total activity rose 73% in 24 hr and subsided to 43% in 48 hr.

A trial with a single injection of GlcCer/Myrj without CBE produced smaller effects, with a 30% increase in total enzyme activity after 24 hr. No significant rise was seen at seven hr.

In vitro tests were run to determine whether the addition of GlcCer could directly stimulate thymidine kinase. Various amounts of GlcCer emulsion (5, 10, 25 and 50 μg/ml) in Tris buffer were added to the incubation mixture just before the addition of liver enzyme. Incubation for 30 or 60 min, with or without GlcCer, produced similar observed activities. In an experiment with 50 μg/ml of GlcCer, with and without 5 μg/ml of phosphatidylserine (from bovine brain, Sigma Chemical Co., St. Louis, MO), with and without a two-hr preincubation, there was also no significant effect of either lipid. However the phosphatidylserine + GlcCer exerted a small inhibitory effect (about 12%). The phospholipid was tested because it is an activator of GlcCer glucosidase.

Another enzyme noted for its responsiveness to various growth promoters, ornithine decarboxylase (9), showed larger responses in activity to GlcCer and CBE (Table 2). This was done as part of the second experiment, above. Again, the liver increase in weight was seen, similar at the two time points. The total activity of ornithine decarboxylase in the stimulated mouse livers was 101% above controls after 24 hr, 64% above after 48 hr.

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Abbreviations: CBE, conduritol B epoxide; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; GlcCer, glucosylceramide.

TABLE 1

Thymidine Kinase Activity in Mice Injected with Glucosylceramide

Measurement	Controls	Treated	Increase (%)
Body wt. (g/mouse)	6.70 (0.38)	6.65 (0.27)	−0.7
Liver wt. (mg/mouse)	277 (20)	327 (27)	18
Protein in liver (mg)	27.6 (1.6)	31.6 (1.1)	14
DNA in liver (μg)	644 (50)	758 (30)	18
Thymidine kinase (μmol/hr)	1.30 (0.09)	1.90 (0.12)	46

Nine Hsd CF1 mice were injected with GlcCer (250 mg/kg emulsified with Myrj, 188 mg/kg) and, a day later, with CBE (80 mg/kg). Saline was injected into nine controls, and all mice were killed 24 hr later. The thymidine kinase activity shown is μmol/hr of thymidine converted to the monophosphate by the cytosol from one mouse liver, less the blank activity from boiled liver. The numbers in parentheses are standard deviations derived from six values (duplicates from three groups of three pooled livers) or nine individual values in the case of the weights. All increases were statistically significant by Student's t-test ($p < 0.005$).

TABLE 2

Ornithine Decarboxylase Activity in Mice Injected with GlcCer and CBE

Measurement	Controls	Treated	Increase (%)
Body wt. 24 hr later (g)	6.71 (0.77)	6.78 (0.46)	0
Body wt. 48 hr later (g)	6.57 (0.66)	6.95 (0.96)	6
Liver wt. 24 hr (mg)	278 (25)	342 (27)	23*
Liver wt. 48 hr (mg)	268 (34)	333 (55)	24*
Ornithine decarboxylase			
24 hr later (nmol/hr)	400 (21)	805 (55)	101*
48 hr later (nmol/hr)	387 (39)	636 (75)	64*

*$p < 0.005$.

The protocol was similar to that shown in Table 1, but both drugs were injected together at time zero, and the animals were killed 24 hr or 48 hr later. Standard deviations are shown in parentheses. Decarboxylase activity shown is the amount of ornithine decarboxylated by one liver in one hr, corrected for the blank activity in the presence of decarboxylase inhibitor.

DISCUSSION

We have shown that an emulsion of GlcCer acts on the liver to stimulate or increase the amount of thymidine kinase, a major enzyme of DNA synthesis (10). The mechanism for the kinase stimulation may not be through a direct action on the enzyme, as indicated by the lack of effect in vitro. However, the activation phenomenon may require other factors that are needed for in vitro stimulation.

The increase in ornithine decarboxylase activity that we observed is consistent with the reported two-fold increase produced by incubating cells with ganglioside, a glycolipid derived from GlcCer (11). The incubated ganglioside probably was hydrolyzed in part to GlcCer, and the GlcCer injected into our mice presumably was converted in part to ganglioside. It remains to be seen which glycolipid or intermediate between them was the actual stimulator. Ornithine decarboxylase formation is induced, generally, by phorbol diester, which apparently acts also to stimulate phosphorylations by a protein kinase C (12) as well as by a second pathway (9). Both pathways apparently activate the formation of ornithine decarboxylase (13). Gangliosides were reported to stimulate a protein kinase C (14) and to exert a proliferative action on cells such as neuroblastoma (15).

Phorbol diester may act through its stimulating effect on glycolipids, exemplified in the finding that it increased the levels of cerebroside, lactosylceramide and ganglioside GM3 in cultured leukemia cells (16). Both GlcCer and phorbol diester increased interleukin formation by macrophage cells (17).

It has been reported that lysosphingolipids are strong inhibitors of protein kinase C and of phorbol diester binding, and the speculation was offered that the lysolipids are responsible for the pathological features of sphingolipidoses such as Gaucher disease (18). Glucosylsphingosine does accumulate in this disorder (albeit to a low concentration) and might be formed to a certain extent in our GlcCer-loaded mice, but one generally cannot conclude that the inhibition of protein kinase C should lead to cell proliferation and increases in enzymes involved in DNA synthesis. Stimulation of protein kinase C leads to increased levels of ornithine decarboxylase (12,13), and we temporarily could conclude that GlcCer injection produces an increase in the kinase, not an inhibition.

In one experiment, we weighed the mouse spleens and found no significant change in weight after injecting GlcCer. The lack of effect can be attributed to our finding of rapid uptake of the exogenous GlcCer by liver (3). This is consistent with an observation made recently with labeled L-glucosylceramide, an unnatural enantiomer that apparently is unmetabolizable (19).

Omitted in this preliminary study was a control group of mice that had been injected only with detergent, instead of saline. Myrj alone had been found to have no effect on liver weight (3), and it has been found to be replaceable by lecithin for production of GlcCer's growth effect (Datta and Radin, unpublished data). Moreover, our experiment with GlcCer/Myrj without CBE produced a smaller increase in thymidine kinase, a result that is consistent with the assignment of the effect to the lipid rather than to the detergent. GlcCer has been found to enhance the proliferation of Ehrlich ascites carcinoma cells in vivo without the inclusion of detergent (20). While it seems highly unlikely that the detergent is responsible for the interesting increases in enzyme activity reported here, future studies should include a detergent or lecithin control group.

We suggest that GlcCer has mitogenic properties, acting through a relatively direct mechanism. It may function not only as a precursor for nearly all the glycolipids but also to control organ growth in normal animals. The possible roles of GlcCer and/or related glycolipids in cell proliferation and cancer have been discussed more fully (21).

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REFERENCES

1. Hara, A., and Radin, N.S. (1979) *Biochim. Biophys. Acta 582*, 423-433.
2. Kanfer, J.N., Legler, G., Sullivan, J., Raghavan, S.S., and Mumford, R.A. (1975) *Biochem. Biophys. Res. Commun. 67*, 85-90.
3. Datta, S.C., and Radin, N.S. (1986) *Lipids 21*, 702-709.
4. Radin, N.S., and Klinger, P. (1986) *Computer Applic. Biosci. 2*, (107-109).
5. Bone, R., Cheng, Y.-C., and Wolfenden, R. (1986) *J. Biol. Chem. 261*, 5731-5735.
6. Cheng, Y.-C. (1978) *Methods Enzymol. 51*, 365-371.
7. Uzumaki, H., Yamamoto, S., and Kato, R. (1986) *Carcinogenesis 7*, 289-294.
8. Kohsaka, S., Schwartz, M., and Agranoff, B.W. (1981) *Dev. Brain Res. 1*, 391-401.
9. Hovis, J.G., Stumpo, D.J., Halsey, D.L., and Blackshear, P.J. (1986) *J. Biol. Chem. 261*, 10380-10386.
10. Kit, S. (1976) *Mol. Cell. Biochem. 11*, 161-182.
11. Roisen, F.J., Bartfeld, H., Nagele, R., and Yorke, G. (1981) *Science 214*, 577-578.
12. Butler, A.P., Byus, C.V., and Slaga, T.J. (1986) *J. Biol. Chem. 261*, 9421-9425.
13. Jetten, A.M., Ganong, B.R., Vandenbark, G.R., Shirley, J.E., and Bell, R.M. (1985) *Proc. Natl. Acad. Sci. USA 82*, 1941-1945.
14. Chan, K.J. (1987) *J. Biol. Chem. 262*, 2415-2422.
15. Tsuji, S., Arita, M., and Nagai, Y. (1983) *J. Biochem. Tokyo 94*, 303-306.
16. Kiguchi, K., Henning-Chubb, C., and Huberman, E. (1986) *Cancer Res. 46*, 3027-3033.
17. Gery, I., Zigler, J.S. Jr., Brady, R.O., and Barranger, J.A. (1981) *J. Clin. Invest. 68*, 1182-1189.
18. Hannun, Y.A., and Bell, R.M. (1987) *Science 235*, 670-674.
19. Tokoro, T., Gal, A.E., Gallo, L.L., and Brady, R.O. (1987) *J. Lipid Res. 28*, 968-972.
20. Inokuchi, J., and Radin, N.S. (1987) *Cancer Lett. 38*, 23-30.
21. Radin, N.S., and Inokuchi, J., *Biochem. Pharmacol.*, in press.

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Absolute Configuration of 12-Oxo-10,15(Z)-phytodienoic Acid

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12-Oxo-10,15(Z)-phytodienoic acid biosynthesized from 13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid using a preparation of corn (*Zea mays* L) hydroperoxide dehydrase recently was found to be a mixture of enantiomers in a ratio of 82:18 (Hamberg, M., and Hughes, M.A. (1988) *Lipids 23,* 469–475). In this work, 12-oxophytodienoic acid and (+)-7-iso-jasmonic acid were converted into a common derivative, methyl 3-hydroxy-2-pentyl-cyclopentane-1-octanoate. From gas liquid chromatographic analysis of the (−)-menthoxycarbonyl derivative of methyl 3-hydroxy-2-pentyl-cyclopentane-1-octanoates prepared from 12-oxophytodienoic acid and (+)-7-iso-jasmonic acid, it could be deduced that the major enantiomer of 12-oxophytodienoic acid had the 9(S),13(S) configuration. Therefore, in the major enantiomer of 12-oxophytodienoic acid, the configurations of the side chain-bearing carbons are identical to the configurations of the corresponding carbons of (+)-7-iso-jasmonic acid, thus giving support to previous studies indicating that 12-oxophytodienoic acid serves as the precursor of (+)-7-iso-jasmonic acid in plant tissue.
***Lipids 23,* 521–524 (1988).**

Conversion of 13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid (13(S)-HPOT) into a cyclopentenone derivative, 12-oxo-10,15(Z)-phytodienoic acid, was reported by Zimmerman and Feng (1). The enzyme activity responsible for the conversion was detected in a wide variety of plants such as wheat, barley, oat, flax, corn, sunflower and lettuce (2). In subsequent work, Vick and Zimmerman demonstrated that 12-oxophytodienoic acid was a precursor of jasmonic acid (3). The transformation was demonstrated in several plant species and consisted of initial saturation of the Δ^{10} double bond of 12-oxophytodienoic acid catalyzed by a reductase followed by three steps of β-oxidation (4). Jasmonic acid has been suggested to act as a plant hormone because of its growth-inhibitory and senescence-promoting effects (5). Recent studies have shown that an epimer of (−)-jasmonic acid, i.e. (+)-7-iso-jasmonic acid, is produced by higher plants (6,7) and fungi (8). Interestingly, in some systems the growth-inhibitory activity of (+)-7-iso-jasmonic acid appears to be higher than that of (−)-jasmonic acid (7).

The absolute configuration of (−)-jasmonic acid was established by Hill and Edwards (9). 12-Oxo-10,15(Z)-phytodienoic acid has been shown to have the *cis* relationship between its two side chains (10) and to consist of enantiomers of unknown configuration in the ratio 82:18 (11). This paper reports the absolute configurations of the major and minor enantiomers of 12-oxophytodienoic acid.

EXPERIMENTAL PROCEDURES

Materials. 13(S)-[1-^{14}C]HPOT (specific radioactivity, 1.5 kBq/μmol) was prepared by incubation of [1-^{14}C]α-linolenic acid (Amersham, UK) with soybean lipoxygenase as described (12). (+)-7-iso-Jasmonic acid (containing ca. 20% of (−)-jasmonic acid) was isolated from the culture medium of the fungus *Botryodiplodia theobromae* as described (8).

Enzyme preparation. A preparation containing corn hydroperoxide dehydrase was obtained by extraction of defatted corn germ (Bear X8632 hybrid corn, Noble Bear, Inc., Decatur, IL) with potassium phosphate buffer followed by ammonium sulfate precipitation (0–45% saturation) as described (13). Precipitate dissolved in ice-cold 0.1 M potassium phosphate buffer, pH 7.4 (1.6 mg of protein per ml buffer) was used for the incubations.

Preparation of methyl 12-hydroxyphytonoates (trans isomers) from (+)-7-iso-jasmonic acid. Epimeric methyl 12-hydroxyphytonoates with defined stereochemistry at carbons 9, 12 and 13 (**4a** and **4b**) were prepared from (+)-7-iso-jasmonic acid (**1**) as shown in Figure 1.

1 (10 mg; containing ca. 20% of (−)-jasmonic acid) was treated with diazomethane and subjected to catalytic hydrogenation. The product was treated with 10 ml of 1 M KOH in 50% aqueous methanol at 60 C for five hr (8). Material isolated by extraction with diethyl ether was subjected to silicic acid chromatography. Elution with diethyl ether/hexane (2:8, v/v) afforded 6 mg of (−)-9,10-dihydrojasmonic acid (**2**). Thin layer chromatographic analysis of this material showed a single spot (solvent

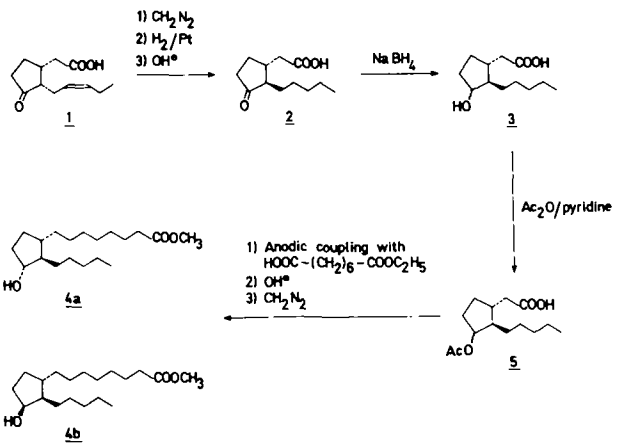

FIG. 1. Preparation of methyl 12(R)-hydroxy-9(S),13(R)-phytonoate (*4a*) and methyl 12(S)-hydroxy-9(S),13(R)-phytonoate (*4b*) from (+)-7-iso-jasmonic acid (*1*).

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Abbreviations: 13(S)-HPOT, 13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid; MC, (−)-menthoxycarbonyl; Me_3Si, trimethylsilyl; GC-MS, gas chromatography-mass spectrometry; GLC, gas liquid chromatography; HPLC, high performance liquid chromatograpy; TLC, thin layer chromatography.

When absolute configurations of C-9 and C-13 are not specifically indicated, phytonoic acid is used to denote 2-pentyl-cyclopentane-1-octanoic acid in which the two side chains have the *cis* relationship, whereas phytonoic acid (*trans* isomer) denotes 2-pentyl-cyclopentane-1-octanoic acid in which the two side chains have the *trans* relationship.

system, ethyl acetate/hexane/acetic acid (4:6:0.05, v/v/v), R_f = 0.50). An aliquot was treated with diazomethane and analyzed by gas chromatography-mass spectrometry (GC-MS). A major peak due to methyl (−)-9,10-dihydrojasmonate (91%) and a minor peak due to methyl (+)-9,10-dihydro-7-iso-jasmonate (9%) were observed. The mass spectrum recorded on the peak of methyl (−)-9,10-dihydrojasmonate showed prominent ions at *m/e* 226 (M, 3%), 195 (M − 31, 3), 156 (M − 70 [β-cleavage with loss of CH_2=CH-C_3H_7], 38), 153 (M − 73 [loss of ·CH_2-$COOCH_3$], 34), and 83 (100).

2 (5 mg) was dissolved in methanol (5 ml) and treated with sodium borohydride (50 mg) at room temperature for 30 min. An aliquot of the product was treated with diazomethane and subjected to thin layer chromatography (TLC) (solvent system, ethyl acetate/hexane [25:75, v/v]). Two spots due to the methyl esters of 6-hydroxy-9,10-dihydrojasmonic acids epimeric at C-6 (**3**) appeared (R_f = 0.33 and R_f = 0.39). The mass spectrum of the trimethylsilyl (Me_3Si) derivative of the methyl ester of **3** showed prominent ions at *m/e* 300 (M, 4%), 285 (M − 15, 27), 226 (M − 74 [loss of ·CH_2-$COOCH_3$ plus H], 20), 210 (M − 90, 8), and 129 (CH_2=CH-CH=O^+ $SiMe_3$, 100).

3 (ca. 4.5 mg) was acetylated by treatment with acetic anhydride (0.3 ml) in pyridine (0.3 ml) at room temperature for 18 hr. Water (10 ml) was added, and the mixture was stirred at room temperature for four hr. An aliquot of the product was treated with diazomethane and subjected to analysis by gas chromatography-mass spectrometry (GC-MS). Two peaks appeared due to the methyl ester of **5** epimeric at C-6. The mass spectra of the two epimers were virtually identical and showed prominent ions at *m/e* 210 (M − 60 [loss of CH_3COOH], 14%), 197 (M − 73 [loss of ·CH_2-$COOCH_3$], 12), 178 (M − [60 + 32], 10), 150 (36), 136 (100), 80 (33), and 74 (CH_2=C[OCH_3]O^+H, 18).

5 (ca. 4 mg) was dissolved in methanol (12 ml) containing ethyl hydrogen suberate (95 mg). One ml of 0.05 M sodium methoxide in methanol was added, and a current of 0.15 A was passed through the solution for three hr. The reaction mixture was treated with 5% of NaOH at room temperature for 18 hr. Material isolated by extraction with diethyl ether was treated with diazomethane and subjected to silicic acid chromatography. The major by-product from the anodic coupling, dimethyl tetradecanedioate, was eluted with diethyl ether/hexane (5:95, v/v). Elution with diethyl ether/hexane (3:7, v/v) afforded methyl 12-hydroxyphytonoate (*trans* isomer, ca. 2 mg). Preparative TLC (solvent system, ethyl acetate/hexane [25:75, v/v]) afforded the pure epimers **4a** (R_f = 0.51) and **4b** (R_f = 0.58). Aliquots were converted into the Me_3Si derivatives and analyzed by GC-MS. The C-values found for **4a** and **4b** were 19.85 and 20.00, respectively, and the mass spectra showed prominent ions at *m/e* 384 (M, 1%), 369 (M − 15, 15), 337 (M − [15 + 32], 9), 294 (M − 90, 10), 137 (M − [157 + 90] [loss of ·$(CH_2)_7$-$COOCH_3$ plus Me_3SiOH], 7), and 129 (CH_2=CH-CH=O^+SiMe_3, 100).

The absolute configuration of C-9 ("S") and C-13 ("R") of **4a** and **4b** followed from their mode of preparation from **1**, the stereochemistry of which is well-established (8). The configuration of the hydroxyl group at C-12 ("R" in **4a** and "S" in **4b**) followed from recent work that has shown that irrespective of the absolute configuration of C-13, the epimer of methyl 12-hydroxyphytonoate (*trans* isomer), which is more polar on TLC analysis, has the *trans* relationship between the hydroxyl group at C-12 and the pentyl side chain attached to C-13, whereas the less polar epimer has the *cis* relationship between the corresponding substituents (11). Accordingly, **4a** was methyl 12(*R*)-hydroxy-9(*S*),13(*R*)-phytonoate, and **4b** methyl 12(*S*)-hydroxy-9(*S*), 13(*R*)-phytonoate (Fig. 1).

Preparation of methyl 12-hydroxyphytonoates (trans isomers) from 12-oxo-10,15(Z)-phytodienoic acid. Epimeric methyl 12-hydroxyphytonoates (**8a** and **8b**, Fig. 2) were prepared from methyl 12-oxo-10,15(*Z*)-phytodienoate (**6**) as described (11). Briefly, 13(*S*)-HPOT was treated with corn hydroperoxide dehydrase at 0 C for 15 min, and the resulting 12-oxophytodienoic acid isolated as its methyl ester (**6**) by TLC and reversed-phase high performance liquid chromatography (HPLC). **7**, prepared by base-catalyzed isomerization of **6** (2 mg), was subjected to sodium borohydride reduction and catalytic hydrogenation. This treatment resulted in the formation of two epimers that were separable by TLC (solvent system, ethyl acetate/hexane [25:75, v/v]; **8a**, R_f = 0.51; **8b**, R_f = 0.58).

As mentioned above, it recently was found that the relative configuration between the hydroxyl group and the pentyl side chain was *trans* in the more polar epimer (**8a**), whereas the relationship between the corresponding substituents was *cis* in the less polar epimer (**8b**) (11). Accordingly, depending on the absolute configuration of **6**, **8a** may be methyl 12(*R*)-hydroxy-9(*S*),13(*R*)-phytonoate and/or methyl 12(*S*)-hydroxy-9(*R*),13(*S*)-phytonoate. In the same way, **8b** may be methyl 12(*S*)-hydroxy-9(*S*),13(*R*)-phytonoate and/or methyl 12(*R*)-hydroxy-9(*R*),13(*S*)-phytonoate (Fig. 2).

Methods for isolation and stereochemical analysis. TLC was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck, FRG. Material was located by spraying with 2′,7′-dichlorofluorescein and viewing under UV light. HPLC was performed using a column (300 × 8 mm) of Polygosil C_{18} 5μ and acetonitrile/water (85:15, v/v) at a flow rate of 1.5 ml/min. The absorbancy of the effluent at 222 nm was measured. Gas liquid chromatography (GLC) was performed with an F&M Biomedical gas chromatograph model 402 using a column of 5% QF-1 on Supelcoport. GC-MS was carried out with an LKB 9000S instrument equipped with a column of 3% OV-210 on Supelcoport.

Methods for catalytic hydrogenation and preparation of Me_3Si derivatives were as described (14). Preparation

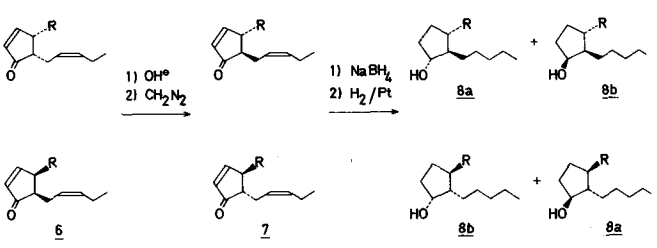

FIG. 2. Preparation of methyl 12-hydroxyphytonoates (*trans* isomers; *8a, 8b*) from methyl 12-oxo-10,15(*Z*)-phytodienoate (*6*). Stereoisomers having the following absolute configurations at C-9 and C-13 are shown: *6*, upper: 9(*S*),13(*S*), lower: 9(*R*),13(*R*); *7*, *8a*, and *8b*, upper: 9(*S*),13(*R*), lower: 9(*R*),13(*S*). R = $(CH_2)_7$-$COOCH_3$.

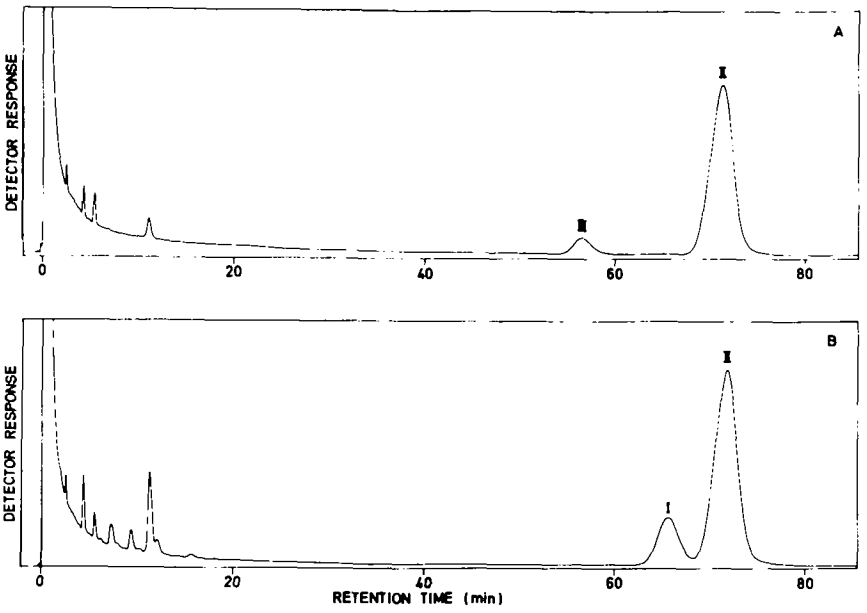

FIG. 3. Gas liquid chromatographic analysis of MC derivatives of *4b* (panel A) and *8b* (panel B). Column, 5% QF-1 on Supelcoport; column temperature, 200 C. I, MC derivative of methyl 12(*R*)-hydroxy-9(*R*),13(*S*)-phytonoate; II, MC derivative of methyl 12(*S*)-hydroxy-9(*S*),13(*R*)-phytonoate; III, MC derivative of methyl 12-hydroxy-9(*S*),13(*S*)-phytonoate.

of (−)-menthoxycarbonyl (MC) derivatives of methyl hydroxyphytonoates were carried out by treating the hydroxy compound (0.05–0.3 mg) with 60 μl of (−)-menthylchloroformate in 40 μl of benzene and 12 μl of dry pyridine at room temperature for 18 hr (15). The MC derivatives were purified by TLC (solvent system, ethyl acetate/hexane [7:93, v/v]) and subjected to gas chromatographic analysis.

RESULTS AND DISCUSSION

In this work, epimeric methyl 12-hydroxyphytonoates (*trans* isomers) of known (**4a** and **4b**) and unknown (**8a** and **8b**) configuration were prepared from (+)-7-iso-jasmonic acid and 12-oxo-10,15(*Z*)-phytodienoic acid, respectively. The epimers were treated separately with (−)-menthylchloroformate, and the resulting MC derivatives were analyzed by GLC. As shown in Figure 3A, the MC derivative of **4b** gave a single peak ("II") confirming that **4b** was optically pure (methyl 12(*S*)-hydroxy-9(*S*),13(*R*)-phytonoate). The minor peak ("III") present in the gas chromatogram was due to the MC derivative of methyl 12-hydroxy-9(*S*),13(*S*)-phytonoate derived from the trace (9%) of (+)-7-iso-jasmonic acid remaining not isomerized after the alkali treatment (see above). Analysis of the MC derivative of **8b**, derived from 12-oxo-10,15(*Z*)-phytodienoic acid, showed two peaks ("II" and "I," ratio 82:18) in agreement with recent work (11) (Fig. 3B). The major peak ("II") coincided with the peak of the MC derivative of **4b** (see Fig. 3; co-chromatography of "II" of the MC derivatives of **4b** and of **8b** was rigorously proved by co-injection of the MC derivatives of **4b** and **8b**). Thus, the major enantiomer of **8b** was methyl 12(*S*)-hydroxy-9(*S*),13(*R*)-phytonoate, and the minor enantiomer (the MC derivative of which gave peak "I") was methyl 12(*R*)-hydroxy-9(*R*),13(*S*)-phytonoate. Analysis of the MC derivatives of **4a** and **8a** gave a similar result, i.e. the MC derivative of the major enantiomer of **8a** co-chromatographed with the MC derivative of **4a** (methyl 12(*R*)-hydroxy-9(*S*),13(*R*)-phytonoate).

The absolute configuration of C-9 of 12-oxo-10,15(*Z*)-phytodienoic acid is unaffected by the treatments shown in Figure 2. Thus, the finding that the configuration of C-9 of the major enantiomers of **8a** and **8b** was "S" coupled with the previous result that the relative configuration of the two side chains of 12-oxo-10,15(*Z*)-phytodienoic acid is *cis* (10) demonstrated that the absolute configurations of C-9 and C-13 of the major enantiomer of 12-oxophytodienoic acid were both "S."

Our finding that the configurations of the chain-bearing carbons of the major enantiomer of 12-oxo-10,15(*Z*)-phytodienoic acid were identical to those of the corresponding carbons of (+)-7-iso-jasmonic acid supports the concept that 12-oxophytodienoic acid serves as the precursor of (+)-7-iso-jasmonic acid in plant tissue (3,4). It was noteworthy, however, that the putative precursor, 12-oxophytodienoic acid, was found to be only 82% optically pure, whereas the product, (+)-7-iso-jasmonic acid, was 100% optically pure. It is possible that 12-oxophytodienoic acid biosynthesized in vivo is optically pure. Another possibility is that 12-oxophytodienoic acid biosynthesized in vivo is, as in this in vitro system, not completely optically pure but that only the major enantiomer can be converted into (+)-7-iso-jasmonic acid. Such a discrimination between enantiomers might be caused by stereospecificity of the reductase that catalyzes saturation of the Δ^{10} double bond of 12-oxophytodienoic acid. Further studies on this problem are in progress and will be reported later.

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REFERENCES

1. Zimmerman, D.C., and Feng, P. (1978) *Lipids 13*, 313-316.
2. Vick, B.A., and Zimmerman, D.C. (1979) *Plant Physiol. 64*, 203-205.
3. Vick, B.A., and Zimmerman, D.C. (1983) *Biochem. Biophys. Res. Commun. 111*, 470-477.
4. Vick, B.A., and Zimmerman, D.C. (1984) *Plant Physiol. 75*, 458-461.
5. Sembdner, G., and Klose, C. (1985) *Biol. Rundsch. 23*, 29-40.
6. Nishida, R., and Acree, T.E. (1984) *J. Agric. Food Chem. 32*, 1001-1003.
7. Miersch, O., Meyer, A., Vorkefeld, S., and Sembdner, G. (1986) *J. Plant Growth Regul. 5*, 91-100.
8. Miersch, O., Preiss, A., Sembdner, G., and Schreiber, K. (1987) *Phytochemistry 26*, 1037-1039.
9. Hill, R.K., and Edwards, A.G. (1965) *Tetrahedron 21*, 1501-1507.
10. Vick, B.A., and Zimmerman, D.C. (1979) *Lipids 14*, 734-740.
11. Hamberg, M., and Hughes, M.A. (1988) *Lipids 23*, 469-475.
12. Hamberg, M., and Gotthammar, B. (1973) *Lipids 8*, 737-744.
13. Hamberg, M. (1987) *Biochim. Biophys. Acta 920*, 76-84.
14. Hamberg, M., Herman, C.A., and Herman, R.P. (1986) *Biochim. Biophys. Acta 877*, 447-457.
15. Hamberg, M. (1971) *Anal. Biochem. 43*, 515-526.

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Formation of a Thiamine Disulfide Complex with Fatty Acid: Mechanism of Formation of the Complex

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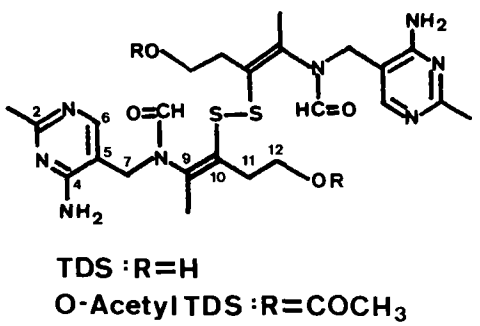
The formation of complexes between thiamine disulfide (TDS) or *O*-acetyl thiamine disulfide (*O*-acetyl TDS) and fatty acid or fatty acid methyl ester in methanol has been studied by fluorescence quenching and ^{13}C NMR relaxation (T_1) measurements. The association constants (K-values) of TDS and *O*-acetyl TDS with fatty acids (from 11:0 to 18:0, and 18:1, 18:2, 18:3 and 20:4) and fatty acid methyl esters have been determined. These values do not depend on either the number of carbon atoms or the degree of unsaturation of the fatty acid. The K-values of TDS and *O*-acetyl TDS with fatty acid were 7.8 M^{-1} and 5.1 M^{-1}, respectively. The K-values of TDS and *O*-acetyl TDS with fatty acid methyl ester were very small. These results show that the -OH moiety in TDS and the -COOH moiety in the fatty acid are necessary for formation of the complex.
***Lipids* 23, 525–527 (1988).**

Many modified thiamine compounds have been synthesized in attempts to increase the pharmacological effect of thiamine (1–3). TDS (thiamine disulfide), an oxidized dimer of thiamine, has been used as an ingredient of pharmaceutical preparations because its absorption and stability are greater than those of thiamine. TDS is readily reduced to thiamine by cysteine, glutathione and other sulfhydryl compounds, so it shows the same biological activity as thiamine in animals.

Complexes of TDS with higher saturated fatty acids were prepared to improve the stability, absorption and taste of TDS in pharmaceutical preparations (4). The molar ratio of TDS to fatty acid in the complexes in the solid state is reported to be 1:6, but the mode of formation of the complexes is unknown.

This paper reports studies on the formation of complexes of TDS with fatty acids in methanol by the fluorescence quenching method and by ^{13}C NMR. The mode of the interaction is discussed.



TDS : R=H
O-Acetyl TDS : R=$COCH_3$

MATERIALS AND METHODS

Thiamine disulfide (hydrated) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4), undecanoic acid (11:0), lauric acid (12:0), tridecanoic acid (13:0), myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0) and heptadecanoic acid (17:0) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of guaranteed reagent grade. TDS-fatty acid complexes were prepared by the method of Ueda et al. (4). Fluorescence spectra were recorded at 25 C with a Hitachi MPF-4 fluorescence spectrophotometer. ^1H NMR and ^{13}C NMR spectra were measured with a JEOL GX-270 NMR spectrometer. The spectra were assigned with reference to Kotera (5), Echols and Levy (6) and Gallo and Sable (7). ^{13}C relaxation times were measured by the inversion recovery method, employing a (180°-τ-90°) pulse sequence (8), in which τ is the delay time between the 180° and 90° pulses. Experimental conditions are described in the legends to Table 3.

O-Acetyl TDS was prepared as follows. TDS (1 g) was dissolved in 10 ml of pyridine and stirred for 15 min. Then acetic acid anhydride (1.04 g) was added slowly with stirring at room temperature for 15 min. The mixture was

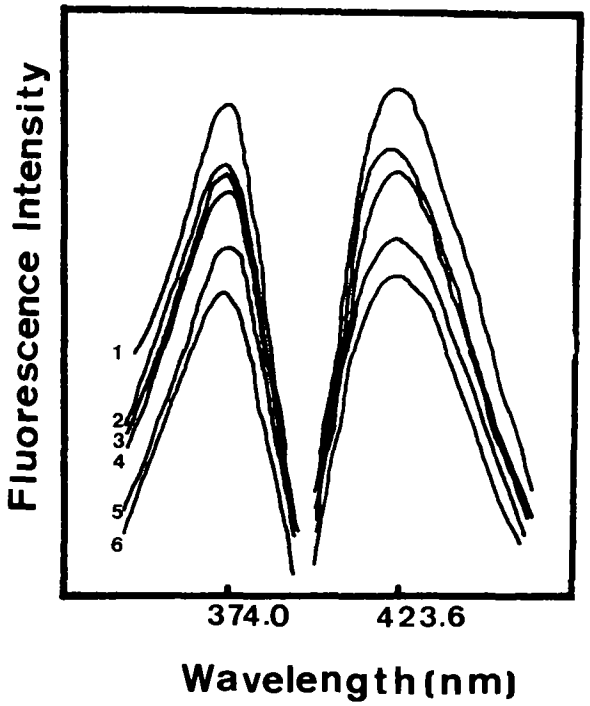


FIG. 1. Fluorescence excitation and emission spectra of 3×10^{-4} M TDS (1) and 3×10^{-4} M TDS with 1, 2, 4, 10 and 20×10^{-3} M oleic acid (2–6) in methanol.

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Abbreviation: TDS, thiamine disulfide.

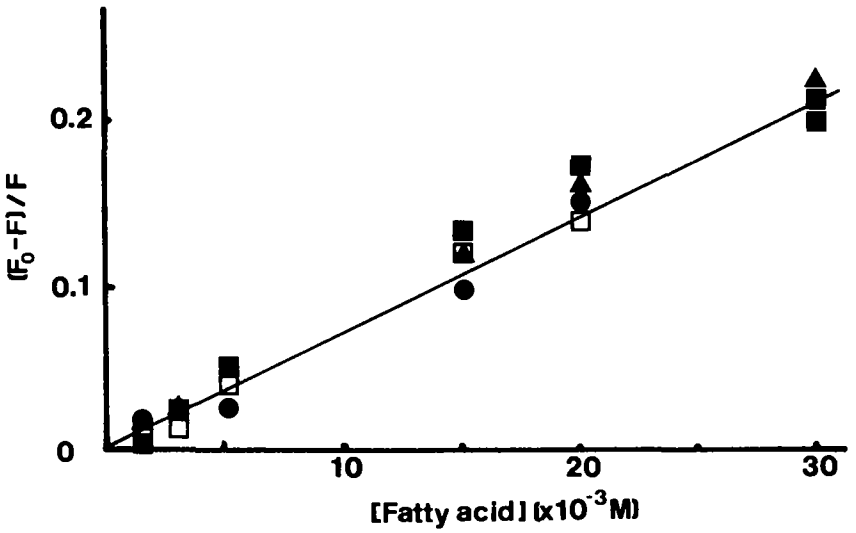

FIG. 2. Stern-Volmer plot of dependence on fatty acid concentration of intensity of TDS fluorescence quenching in methanol. TDS: 3×10^{-4} M, λex = 374.0 nm, λem = 423.6 nm, spectral slit width = 10 nm. Fatty acid: 18:0, ●; 18:1, ■; 18:2, □; 18:3, ▲.

poured onto ice, extracted with chloroform (3 × 20 ml) and washed with saturated sodium bicarbonate solution and water. The chloroform extract was dried with sodium sulfate and evaporated under reduced pressure at 50–60 C. The residue was triturated with ether to yield *O*-acetyl TDS as a white powder (0.45 g, 51.8% yield), m.p. 120–121 C. TLC: ($CHCl_3$/MeOH, 2:1) Rf 0.9. IR (KBr): 3350 (ν_s NH_2), 2930 (ν_s CH_2, CH_3), 1731 (O-CO-CH_3), 1670 (-N-C=O), 1240 cm^{-1} (-C-O-C). UV λmax (CH_3OH): 272.0 (ε; 8300), 233.5 (ε; 18900) nm. MS (m/e): 647 (M^+), 605 ($M^+ - CH_3CO$), 563 ($M^+ - 2x$ [$COCH_3$]). ^1H NMR ($CDCl_3$): δ 2.02 ppm (*s*, 3H, 9-C<u>H</u>$_3$), 2.04 (*s*, 3H, 2-C<u>H</u>$_3$), 2.45 (*s*, 3H, acetyl C<u>H</u>$_3$), 2.55 (b*t*, 2H, C<u>H</u>$_2$-CH_2), 4.05 (*t*, 4H, J = 8.1 Hz, C<u>H</u>$_2$O, C<u>H</u>$_2$N), 6.35 (bs, 2H, N<u>H</u>$_2$), 7.80 (*s*, 2H, C<u>H</u>O, NC<u>H</u>). ^{13}C NMR ($CDCl_3$): δ 170.57 ppm (*s*, acetyl <u>C</u>O), 168.03 (*s*, C-2), 162.96 (*d*, N-CHO), 162.18 (*s*, C-4), 156.11 (*d*, C-6), 135.25 (*s*, C-9), 133.09 (*s*, C-10), 107.76 (*s*, C-5), 61.35 (*t*, C-12), 40.14 (*t*, C-7), 29.49 (*t*, C-11), 25.44 (*q*, 2-C<u>H</u>$_3$), 20.72 (*q*, acetyl C<u>H</u>$_3$), 18.40 (*q*, 9-C<u>H</u>$_3$).

RESULTS AND DISCUSSION

We used the fluorescence quenching technique to study complex formation, as reported (9). When the TDS-fatty acid complex was dissolved in methanol, the fluorescence intensity of TDS increased as the time proceeded, concomitant with the dissociation of the TDS-fatty acid complex. The excitation and emission spectra of TDS fluorescence in methanol before and after addition of increasing concentrations of fatty acids are shown in Figure 1. Addition of fatty acid to the TDS in methanol solution resulted in a decrease in fluorescence intensity (Ex. 374.0 nm, Em. 423.6 nm), due to interaction of TDS with the fatty acid. Over a wide range of fatty acid concentrations, the reaction conformed to the equation of Stern-Volmer (10): $(F_0 - F)/F = K[Q]$, in which F_0 and F denote the fluorescence intensities in the absence and presence of a fatty acid, respectively, and [Q] the concentration of a fatty acid (Fig. 2). The K-value for the interaction of TDS with fatty acid was calculated to be about 7.8 M^{-1} (Table 1). This K-value did not depend on the number of carbon atoms from 11 to 20 or the degree of unsaturation of the fatty acid.

Addition of a fatty acid methyl ester to TDS in methanol resulted in smaller changes in intensity of fluorescence than addition of a free fatty acid, and the K-values of TDS for fatty acid methyl esters were smaller than those for free fatty acids (Table 2). These results show that the -COOH moiety of the fatty acid interacts with TDS to form a complex.

TABLE 1

Association Constants (K) for Interaction of TDS with Fatty Acids in Methanol Calculated from Stern-Volmer Coordinates

Fatty acid	K(M^{-1})	Fatty acid	K(M^{-1})
11:0	5.9 ± 3.1	17:0	7.2 ± 3.2
12:0	9.6 ± 2.3	18:0	7.3 ± 2.6
13:0	9.6 ± 5.1	18:1	7.0 ± 1.9
14:0	6.4 ± 3.9	18:2	6.4 ± 3.7
15:0	7.9 ± 3.5	18:3	8.8 ± 1.6
16:0	8.5 ± 3.6	20:4	9.2 ± 5.4

TABLE 2

Values of the Constant (K) for Interaction of TDS and *O*-Acetyl TDS with Fatty Acid and Fatty Acid Methyl Ester in Methanol Calculated from Values for Fluorescence Quenching

	K(M^{-1})
TDS + FA	7.8 ± 1.3
TDS + FMe	1.9 ± 0.9
*O*Ac + FA	5.1 ± 1.3
*O*Ac + FMe	2.0 ± 0.8

*O*Ac, *O*-Acetyl TDS; FA, fatty acid; FMe, fatty acid methyl ester.

TABLE 3

Changes in the ^{13}C-Spin Lattice Relaxation Time (T_1)[a] of TDS[b] with Stearic Acid Concentration

Position	TDS carbon (ppm)	Added stearic acid (mg)			
		0	1.0	3.0	5.0
10	135.7	3.07	2.61	2.56	2.30
5	110.1	3.13	3.41	3.03	2.75
12	60.5	0.42	0.41	0.40	0.38
7	40.8	0.32	0.23	0.23	0.19

[a]Sec.

[b]50 mg in 1.0 ml methanol-d_4, 27.0 C.

Pulse delay = 10 sec. Pulse interval = 6.0, 3.0, 2.5, 1.8, 0.8, 0.6, 0.4, 0.2, 0.1 and 0.05 sec.

Using the same method, we found that the K-values of *O*-acetyl TDS for fatty acids were smaller than those of TDS. The K-value of *O*-acetyl TDS for fatty acid methyl ester was quite small and similar to that of TDS (2.0 M^{-1} and 1.9 M^{-1}, respectively). These results suggest that the -OH moiety of TDS interacts with the -COOH moiety of the fatty acid.

We measured the ^{13}C spin-lattice relaxation time (T_1) of each carbon atom of TDS in the complex by ^{13}C-NMR spectroscopy. As can be seen in Table 3, the T_1-values of the carbon atoms at positions 10, 5, 12 and 7 of TDS in methanol were reduced progressively by increasing concentrations of stearic acid, but little change was observed in the T_1-values of other carbon atoms. The reduction in the T_1-values was due to restricted mobility of the carbon atoms in TDS by the formation of a complex. The results obtained by ^{13}C NMR T_1 measurement support those obtained by the fluorescence quenching method.

We conclude from this work that TDS forms a complex with fatty acids in methanol and that formation of this complex is not affected by the number of carbon atoms or the degree of unsaturation of the fatty acid. The -OH moiety in TDS and the -COOH moiety in the fatty acid are concluded to be necessary for this interaction. We now are investigating another mode of interaction of TDS with fatty acids.

REFERENCES

1. Zima, O., Götterman, G., Hoffman, A., Hepding, L., and Hotony, G. (1953) *E. Merk's Jahresber 67,* 1-58.
2. Utsumi, I., Harada, K., Kobayashi, H., Kohno, K., Yasuda, K., Kondo, Y., and Hirao, H. (1962) *Vitamins 25,* 69-74.
3. Utsumi, I., Harada, K., Kobayashi, H., Kohno, K., Yasuda, K., Kondo, Y., and Hirao, H. (1962) *J. Vitaminol. 8,* 213-219.
4. Ueda, F., Higashi, T., Ayukawa, Y., Takada, A., Fujie, T., and Kaneko, A. (1987) *Vitamins 61,* 57-64.
5. Kotera, K. (1965) *Chem. Pharm. Bull. 13,* 440-443.
6. Echols, E.R., and Levy, C.G. (1974) *J. Org. Chem. 39,* 1321-1322.
7. Gallo, A.A., and Sable, Z.H. (1974) *J. Biol. Chem. 249,* 1382-1389.
8. Vold, R.L., Waugh, J.S., Klein, M.P., and Phelps, D.E. (1968) *J. Chem. Phys. 48,* 3381-3386.
9. Urano, S., and Matsuo, M., (1987) in *Clinical and Nutritional Aspects of Vitamin E* (Hayaishi, O., and Mino, M., eds.) pp. 281-284, Elsevier Publishing Co., Amsterdam.
10. Stern, O., and Volmer, M. (1919) *Z. Phys. 20* 183-188.

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Purification and Characterization of Fatty Acid-binding Protein from Human Placenta

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Purification of a cytosolic fatty acid-binding protein (FABP) from developing human placenta has been achieved, and its role in modulating the inhibition of human placental glucose-6-phosphate dehydrogenase (G6PD) by palmitoyl-CoA (PAL-CoA) has been studied. FABP was resolved into three peaks, viz. DE-I, DE-II and DE-III, by DEAE cellulose chromatography. DE-I was almost lipid-free. Presence of endogenous fatty acids in DE-II and DE-III was detected by thin layer chromatography (TLC). Fatty acids were the only detectable lipid component in these fractions. Gas liquid chromatography (GLC) analysis revealed that DE-II binds long chain saturated and unsaturated fatty acids nonspecifically, whereas DE-III is mainly an arachidonic acid carrier. Each of these fractions, viz. DE-I, DE-II and DE-III, has a molecular weight of 14,200 Daltons. Ouchterlony double immunodiffusion studies have confirmed the immunochemical identity of these three fractions of placental FABP. Separation in ion exchanger may be due to their different isoelectric points and varied types of binding affinities. Human placental G6PD was inhibited 50% by 0.03 mM PAL-CoA. The DE-II fraction of FABP enhanced the activity of G6PD in the absence of added PAL-CoA and protected against PAL-CoA inhibition of the enzyme. Such a modulating effect of FABP in this inhibition is attributable to binding of long chain acyl-CoA rather than to a direct effect of FABP on the enzyme itself.
Lipids 23, 528–533 (1988).

The presence of low molecular weight fatty acid-binding proteins (FABP) in the cytosol of various animal tissues was first reported by Ockner et al. (1). FABP appears to be identical with Z protein (2) and sterol carrier protein (3). This protein has been purified from many tissues (4,5). It binds free fatty acids, their CoA derivatives, organic anions and other small molecules (6). In 1985, Gordon et al. (7) found the tissue-specific expression and developmental regulation of two genes coding for rat liver and intestinal FABP; these two proteins were immunochemically unrelated. However, both human and rat liver FABP were reported to be highly homologous (8). Trulzsch and Arias observed that in a specific tissue, FABP may exist in more than one form that differ in isolectric points and binding affinities but are immunochemically identical (9). FABP is also required for ATP/ADP transport (10) across the membranes. Burnett et al. (11) have raised the possibility that this protein affects the metabolic compartmentalization of fatty acids between oxidative and esterification pathways. FABP protects many enzymes of lipid synthesis, e.g. acetyl-CoA carboxylase (12), fatty acid synthase (13), ATP citrate lyase (13), citrate synthetase (14), etc., against inhibition by long chain acyl-CoA. It has been suggested that long chain fatty acids and fatty acyl-CoA may be compartmentalized in the cell and that changes in their distribution might affect these enzymes and lipogenesis out of proportion to any detectable change in their tissue concentration. Jacobs and Majerus (15) and Halestrap and Denton (16) suggested that intracellular compartmentation might be affected in part by their binding to FABP. The present experiments have been designed to purify and characterize FABP from developing human placenta and to investigate the hypothesis that interaction between FABP and long chain acyl-CoAs modulates the activity of glucose-6-phosphate dehydrogenase (G6PD), the key enzyme of the hexose monophosphate (HMP) shunt pathway, which provides NADPH for lipid biosynthesis (17) and is inhibited by fatty acyl-CoA (18).

MATERIALS AND METHODS

12-(9-Anthroyl)stearic acid, palmitoyl-CoA (PAL-CoA), glucose-6-phosphate (G6P), nicotinamideadenine dinucleotide phosphate (NADP), α-lactalbumin, soybean trypsin inhibitor, trypsinogen, carbonic anhydrase, cytochrome c, sodium dodecyl sulfate (SDS), Tris (hydroxymethyl)-amino methane (Tris), diethylaminoethyl (DEAE) cellulose, agarose and 1-ethyl-3(-3-dimethylaminopropyl)carbodimide (EDAC) were purchased from Sigma Chemical Co. (St. Louis, MO). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were obtained from Difco Chemicals (Detroit, MI). All other chemicals were of analytical grade and were purchased from local dealers.

Human placentas of gestational ages between 6–30 wk were collected from patients undergoing legal abortion either by suction or via hysterotomy and term placentas were collected at the time of parturition or via Cesarean section from the Department of Obstetrics and Gynecology, National Medical College and Hospital (Calcutta, India). Tissues were collected within 15 min of operation/delivery and kept on ice. Gestational ages were calculated from the period of amenorrhea and by weight and crown-rump length of the fetus (19).

Purification of FABP from human placenta. FABP from human placenta were purified by modification of the procedure published by Das and Mukherjea (20). Placentas were dissected and cut into small segments and rinsed with 0.9% saline to remove blood as much as possible. These segments were then homogenized in 0.01 M Tris-HCl buffer, pH 8.5, by seven passes of a Teflon glass homogenizer. The homogenate was centrifuged for 30 min at 36,000 × g. The supernatant was then spun at 105,000 × g for one hr. The clear cytosol was heated at

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Abbreviations: DEAE, diethylaminoethyl; EDAC, 1-ethyl-3(-3-dimethylaminopropyl)-carbodimide; FABP, fatty acid-binding protein; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; HMP, hexose monophosphate; NADP, nicotinamide-adenine dinudeotide phosphate; PAL-CoA, palmitoyl-CoA; SDS, sodium dodecyl sulfate; Tris, tris (hydroxymethyl)amino methane; CFA, complete Freund's adjuvant; GLC, gas liquid chromatography; IFA, incomplete Freund's adjuvant; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography.

50 C for 20 min and shaken vigorously with 25% butanol for one min. Then, centrifugation was done at 36,000 × g for 30 min to remove the lipids and denatured proteins. The delipidated cytosol was lyophilized to remove butanol completely. Dry powder thus obtained was dissolved in 0.01 M Tris-HCl buffer, pH 8.5, and incubated with 12-(9-anthroyl)stearic acid dissolved in butanol at 37 C for 30 min. This sample was chromatographed on a Sephacryl S-200 column (2.5 × 60 cm, flow rate 25 ml/hr) equilibrated with the same buffer. Fractions of 4.5 ml each were collected and assayed for protein and 12-(9-anthroyl)-stearic acid. Those corresponding to the second peak were pooled and lyophilized. The dry powder was dissolved in the same buffer and chromatographed on a DEAE cellulose column (1.5 × 10 cm) equilibrated with a linear gradient of NaCl up to 0.3 M in 0.01 M Tris-HCl, pH 8.5. Fractions of 2 ml each were collected and assayed at 280 nm for protein and at 364 nm for 12-(9-anthroyl)-stearic acid in a Hitachi Spectrophotometer (Model No. 200-20). SDS gel electrophoresis was carried out according to the method of Laemmli and Favre (21). In other experiments, placental cytosol was lyophilized without prior heat treatment and butanol precipitation. Dry powder then was dissolved in 0.01 M Tris-HCl buffer, pH 8.5, and chromatographed on a Sephacryl S-200 column (2.5 × 60 cm, flow rate 25 ml/hr) equilibrated with the same buffer. Fractions of 4.5 ml each were collected and assayed for the proteins of placental supernatant.

Lipid analysis. Lipids were extracted from native and butanol-treated samples by the method of Folch et al. (22) and were separated by thin layer chromatography (TLC) (0.25 mm Silica Gel 60 in petroleum ether/diethyl ether/glacial acetic acid, 80:20:1). Appropriate zones were identified by means of standards and were extracted from the chromatogram. For analysis of covalently bound fatty acids, the protein was extracted three times with chloroform/methanol (1:1) and then hydrolyzed in 5.7 M HCl at 110 C for 24 hr under vacuum. Free fatty acids were extracted from the hydrolysate as described above. Fatty acid methyl esters were prepared by heating at 80–90 C for two hr in methanol/benzene/sulfuric acid (86:10:4) (23) and were separated by gas liquid chromatography (GLC) on a 6 ft × ⅛ in column of 10% diethylene glycol succinate on 100–120 chromosorb WHP using a Hewlett Packard Chromatograph equipped with a flame ionization detector. Pure methyl esters were used as standards.

Immunological studies. For raising antibody, the DE-II fraction of FABP was dialyzed extensively against distilled water and lyophilized. The homogeneity of the DE-II preparations was checked by polyacrylamide gel electrophoresis (PAGE) under nondenaturing and denaturing conditions. Monomeric DE-II was found to be very weakly immunogenic, thus this protein was polymerized with EDAC. Rabbits were immunized with two subcutaneous injections of polymerized DE-II in 0.9% saline emulsified in CFA (1:1) given at an interval of 10 days (0.5 mg of DE-II/injection), followed by one injection with IFA. The immunesera was collected about 10 days after the last injection. Specificity of antiserum and immunochemical identity of antigens in various FABP fractions as well as in placental cytosol was assayed by Ouchterlony double immunodiffusion method (24).

Preparation and assay of G6PD. Human placentas were dissected, homogenized in a glass homogenizer in 0.25 M sucrose to obtain 20% homogenate and centrifuged at 105,000 × g for one hr at 0–4 C. The enzyme activity was measured in the cytosolic portion according to the method of Löhr and Waller (25) following the increase in absorbance at 340 nm. Protein was estimated according to the method of Lowry et al. (26).

RESULTS

Purification of FABP. In an earlier communication by us, the presence of FABP in human placenta throughout the gestation has been indicated (20). Purification of FABP from placentas of all gestations is being reported in this

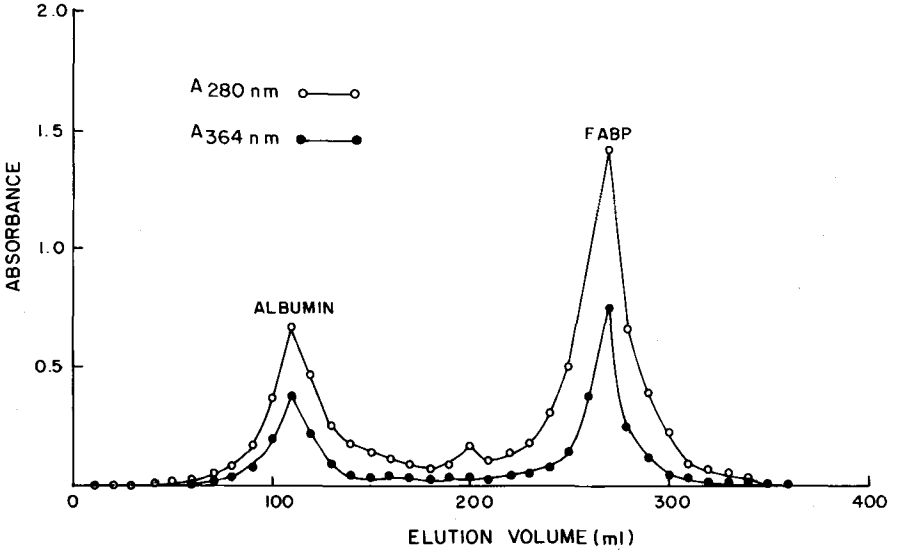

FIG. 1. Gel filtration of human placental cytosolic proteins on Sephacryl S-200. The protein sample (O) after heat treatment and delipidation was charged with 12-(9-anthroyl)stearic acid (●) as described under Materials and Methods and fractionated on a Sephacryl S-200 column (2.5 × 60 cm) equilibrated with 0.01 M Tris-HCl, pH 8.5.

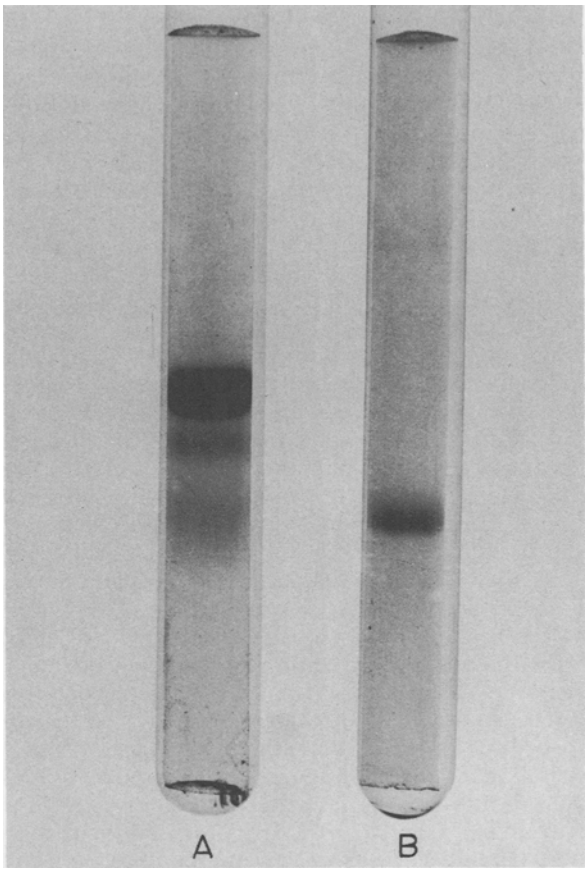

FIG. 2. Ten percent polyacrylamide gel electrophoresis of human placental FABP after Sephacryl S-200 fractionation; (A) under nondenaturing condition; (B) in presence of SDS.

paper. Because an almost similar pattern has been found for FABP in each gestation, results of mid-gestation, i.e. 18–22 wk, are shown here.

When the delipidated cytosolic proteins from human placenta were charged with 12-(9-anthroyl)stearic acid and separated by chromatography on Sephacryl S-200, the fatty acid emerged from the column in two peaks (Fig. 1). The first peak corresponds to serum albumin according to its position. The second peak was due to FABP. These fractions showing three bands in polyacrylamide gel electrophoresis and a single band in polyacrylamide gel electrophoresis in presence of SDS (Fig. 2) were further resolved into three peaks by DEAE cellulose chromatography (Fig. 3). The first of these, i.e. DE-I, was absorbed weakly to the DEAE column and eluted with the equilibrating buffer (0.01 M Tris-HCl, pH 8.5). DE-II and DE-III were eluted with a linear gradient of NaCl up to 0.3 M in the same buffer. DE-I and DE-III showed virtually no binding with 12-(9-anthroyl)stearic acid, but DE-II showed considerable binding. The presence of endogenous fatty acids in DE-II and DE-III (without delipidation or addition of exogenous fatty acid) was detected by TLC after lipid extraction (figure not shown here). GLC analysis revealed that DE-II binds long chain fatty acids nonspecifically, but DE-III is mainly an arachidonic acid binder (Table 1). However, long chain fatty acids were the only detectable lipid component in these fractions and were completely extracted by organic solvent and not detected in the acid hydrolysate of residue proteins. Each of the three fractions gave a single band (Fig. 4) and had an almost similar R_f value on polyacrylamide gel electrophoresis in presence of SDS, which corresponds to the molecular weight of 14,200 Daltons (Fig. 4). When the placental cytosol (without prior heat

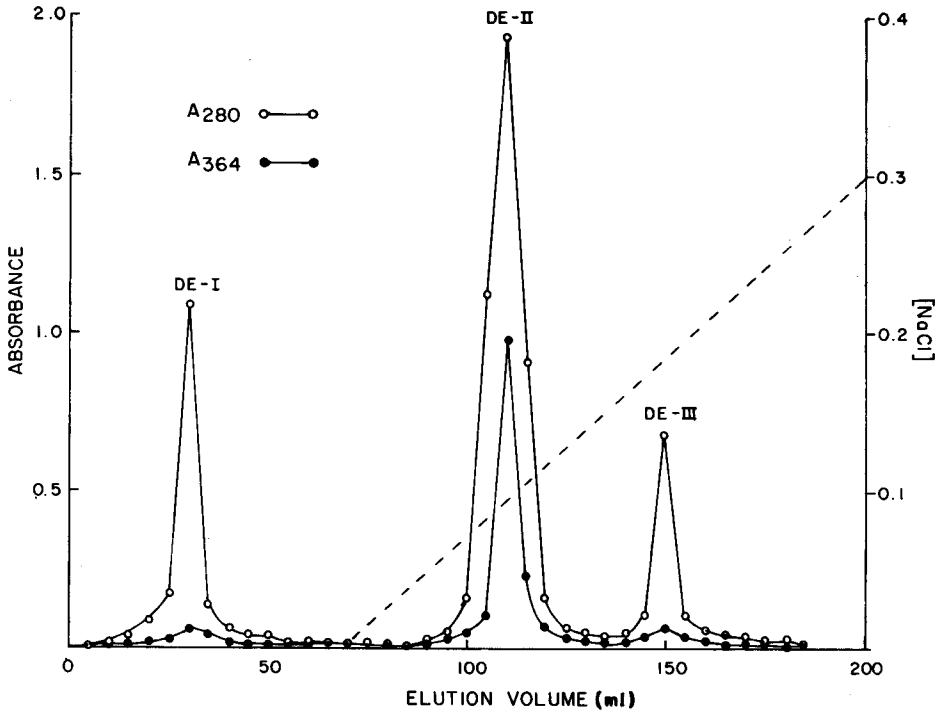

FIG. 3. Chromatography of FABP on DEAE cellulose. Protein concentration (O) and 12-(9-anthroyl)stearic acid (●). Elution was achieved with a linear gradient of NaCl up to 0.3 M in 0.01 M Tris-HCl, pH 8.5.

TABLE 1

Endogenous Fatty Acid Composition of Different FABP Fractions from Human Placental Cytosol

FABP fractions	Bound fatty acids	
DE-I	Virtually lipid-free	
DE-II	Myristic acid	9.62%
	Palmitic acid	19.64%
	Stearic acid	13.99%
	Oleic acid	26.61%
	Linolenic acid	30.12%
DE-III	Palmitic acid	20.52%
	Stearic acid	4.61%
	Oleic acid	10.53%
	Arachidonic acid	64.20%

treatment or butanol precipitation) was chromatographed on Sephacryl S-200, five peaks were obtained (Fig. 5). The fifth peak corresponds to FABP. Endogenous fatty acids were detected in FABP, and in the second peak, which corresponds to serum albumin, by TLC after lipid extraction. The other three peaks were virtually lipid-free. DE-II was employed as an antigen for preparation of an antiserum to FABP. This antiserum produced a single immunoprecipitation line of identity against placental supernatant (Fig. 6A). It can be seen that there were identity reactions between this line and that formed with DE-I, DE-II and DE-III (Fig. 6B, C and D).

Regulation of palmitoyl-CoA inhibition of G6PD by FABP. G6PD activity was found throughout the gestations so far studied. As high activity was associated with 10–15 wk of gestation, further studies have been carried out with tissues of this period. Human placental G6PD was found to be inhibited by PAL-CoA (Fig. 7A). In the experiments shown in curves a, b and c, the reactions were started by the addition of the enzyme. Nearly 50% inhibition was obtained by 0.03 mM PAL-CoA, and almost

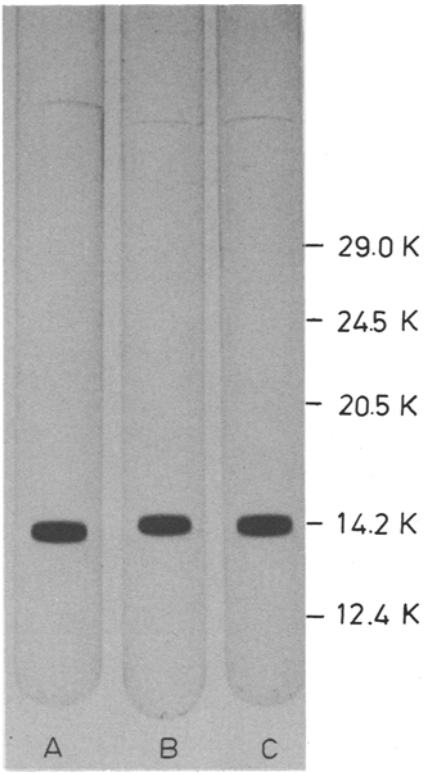

FIG. 4. Ten percent polyacrylamide gel electrophoresis in the presence of SDS. Electrophoresis was carried out at pH 8.3. A, DE-I; B, DE-II; C, DE-III.

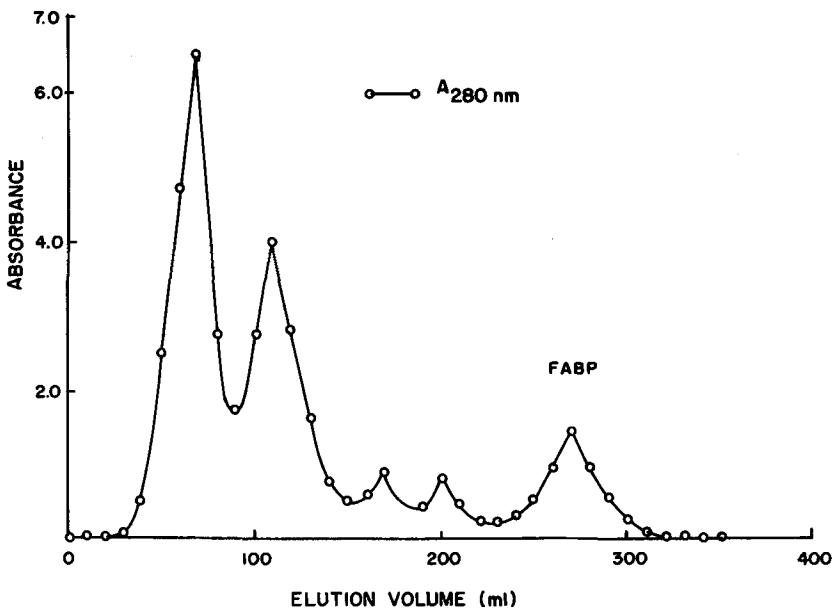

FIG. 5. Gel filtration of human placental cytosolic proteins on Sephacryl S-200. The protein sample (O), without any heat treatment or butanol precipitation, was fractionated on a Sephacryl S-200 column (2.5 × 60 cm) equilibrated with 0.01 M Tris-HCl, pH 8.5.

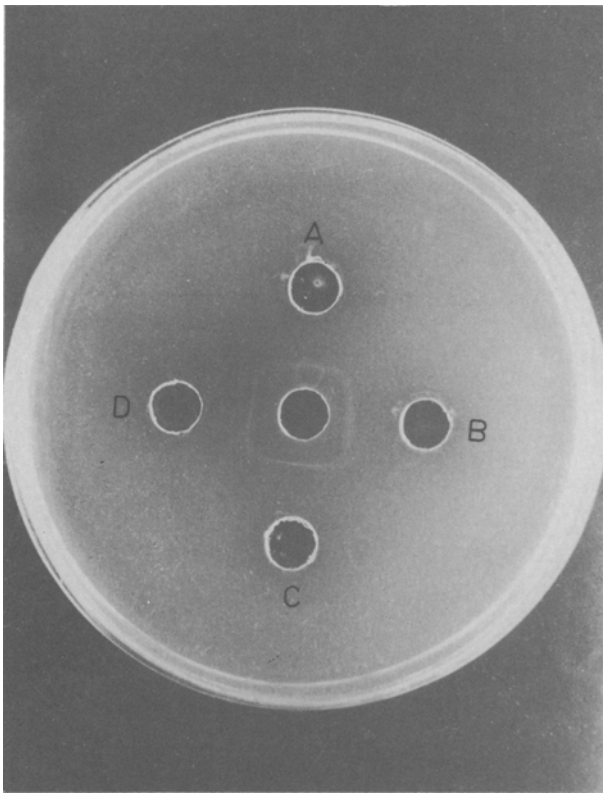

FIG. 6. Ouchterlony double immunodiffusion. Central well contained antisera against DE-II (0.84 mg). A, Human placental supernatant (40 μg); B, DE-I (10 μg); C, DE-II (10 μg); D, DE-III (10 μg).

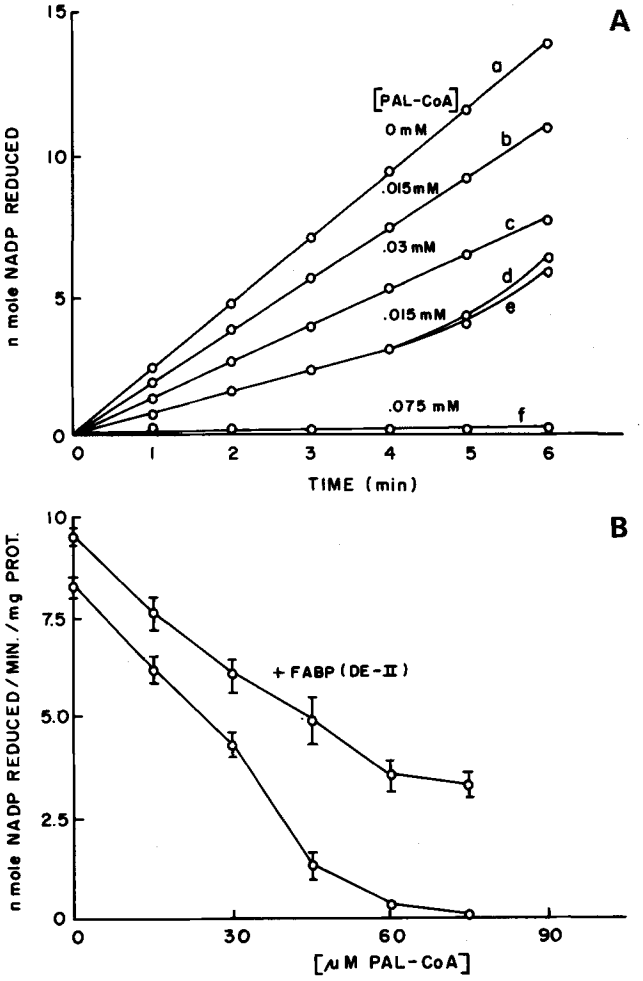

FIG. 7. (A) Inhibition of human placental G6PD by PAL-CoA. The assay mixture contained 0.05 M triethanolamine buffer, pH 7.5, 0.13 mM G6P, 0.1 mM NADP, 0.25 μg/ml of enzyme and PAL—CoA as indicated in a total volume of 3.0 ml. Reaction was started by the addition of enzyme (curves a, b, c and f). The enzyme was preincubated for three min with PAL-CoA and either NADP (curve b) or G6P (curve d), and the reaction was started by the addition of the other substrate. In curve e, the enzyme was preincubated for three min with PAL-CoA alone. (B) Effect of FABP on human placental G6PD activity. Assay mixture contained 0.05 M triethanolamine buffer, pH 7.5, 0.13 mM G6P, 0.1 mM NADP, 0.25 μg/ml enzyme and PAL-CoA as indicated in a total volume of 3 ml. Concentration of FABP was 25 μg/ml. Each point is the mean ± SEM of triplicate experiments.

complete inhibition was obtained with 0.075 mM PAL-CoA (curves c and f, respectively). When the enzyme was preincubated with PAL-CoA and NADP, the extent of inhibition was the same as that shown in curve b. However, when the enzyme and PAL-CoA were preincubated with either G6P alone (curve d) or without any substrate (curve e), inhibition was more severe for the first five min. Thereafter, less inhibition was observed. These results show that NADP but not G6P protects the enzyme against PAL-CoA inhibition.

It is evident from Figure 7B that DE-II reversed the inhibition of G6PD by PAL-CoA. When DE-II was added in absence of any exogenous PAL-CoA, the enzyme was stimulated. Other cytosolic proteins of the placenta, which did not bind endogenous fatty acids, have no effect on the inhibition of G6PD by PAL-CoA.

DISCUSSION

Results of the separation of FABP into three fractions by DEAE cellulose chromatography conform with those reported by Takahashi et al. (27). Human placental FABP can be grouped into at least three distinct fractions with respect to their lipid content (Table 1), i.e. DE-I (almost lipid-free), DE-II (binds long chain fatty acids nonspecifically) and DE-III (binds arachidonic acid mainly). Purification of DE-III from human placenta is of particular importance because arachidonic acid is known to be a common precursor of various prostaglandins, but it cannot be synthesized de novo inside the fetal tissues. Thus, it is mandatory for the fetus to import arachidonic acid from the maternal source through the placenta, and it has been reported that the concentration of this polyunsaturated fatty acid is high in fetal blood (28). DE-I, DE-II and DE-III were found to contain no detectable phospholipids, suggesting that they are not likely to be involved in phospholipid transfer or exchange. These proteins bind ligands noncovalently because long chain fatty acids were completely extracted by organic solvent and not detected in the acid hydrolysate of the residue proteins.

From the Ouchterlony double immunodiffusion studies, it is evident that human placental FABP exists in several immunochemically identical forms, differing in amount

and nature of bound endogenous fatty acids and in isoelectric points. That bound ligands may influence the charge of the carrier molecule is shown by the effect of the bound fatty acid on the pI of serum albumin (29). Other possible explanations for the existence of charge isoforms of FABP are not excluded.

Inhibition of G6PD by PAL-CoA has been proposed as the possible regulatory mechanism for lipogenesis and ketogenesis (13). Because NADP protects the dehydrogenase against PAL-CoA inhibition (Fig. 7A), the binding sites for the two ligands cannot be identical. According to Kawaguchi and Bloch (18), PAL-CoA produces a conformationally altered dehydrogenase with lowered affinity for NADP. The destabilized, coenzyme-depleted tetramer then dissociates to the dimer with exposure of high affinity sites for PAL-CoA. Displacement of PAL-CoA from the inactive enzyme by human placental FABP, which has a high affinity for the inhibitor, regenerates the dehydrogenase tetramer. FABP can reverse the inhibition by removing the inhibitor rather than affecting the enzyme directly. In absence of any exogenous PAL-CoA, G6PD is activated by FABP, indicating the binding of endogenous inhibitors by the latter. These results may explain why these inhibitory effects of long chain fatty acyl-CoA are not seen in intact cells. Such regulation of G6PD by FABP is important because this enzyme is the main source of NADPH for lipid biosynthesis during embryogenesis.

The binding properties and widespread tissue distribution of FABP suggest that it may participate in the potentially critical aspects of cellular physiology and may serve as intracellular analogues of albumin (27), affecting the transport and compartmentation of long chain fatty acids and fatty acyl-CoA inside the cells. Further work is underway to study the structure and function relationship of DE-I, DE-II and DE-III to find out their exact roles in lipid transport and metabolism during human embryogenesis. A possible role in carcinogenesis has been postulated for FABP (30). Hence, characterization of these proteins is of utmost importance for the safety of the unborn child.

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REFERENCES

1. Ockner, R.K., Manning, J.A., Poppenhousen, R.B., and Ho, W.K.L. (1972) *Science 177*, 56–58.
2. Levi, A.J., Gatmaitan, Z., and Arias, I.M. (1969) *J. Clin. Invest. 48*, 2156–2167.
3. Dempsey, M.E., McCoy, K.E., Baker, H.N., Dimitriadou-Vafiadou, A., Lorsbach, T., and Howard, J.B. (1981) *J. Biol. Chem. 256*, 1867–1873.
4. Ockner, R.K., Manning, J.A., and Kane, J.P. (1982) *J. Biol. Chem. 257*, 7872–7878.
5. Said, B., and Schulz, H. (1984) *J. Biol. Chem. 259*, 1155–1159.
6. Fleischner, G.M., and Arias, I.M. (1976) in *Progress in Liver Disease* (Popper, H., and Schaffner, F., eds.) Vol. 5, pp. 172–182, Grune & Stratton, New York.
7. Gordon, J.F., Elshourbagy, N., Lowe, J.B., Liao, W.S., Alpers, D.H., and Taylor, J.M. (1985) *J. Biol. Chem. 260*, 1995–1998.
8. Chan, L., Wei, C., Li, W., Yang, C., Ratner, P., Pownall, H., Gotto, A.M. Jr., and Smith, L.C. (1985) *J. Biol. Chem. 260*, 2629–2632.
9. Trulzsch, D., and Arias, I.M. (1981) *Arch. Biochem. Biophys. 209*, 433–440.
10. Barbour, R.L., and Chan, S.H.P. (1979) *Biochem. Biophys. Res. Commun. 89*, 1168–1177.
11. Burnett, D.A., Lysenko, N., Manning, J.A., and Ockner, R.K. (1979) *Gastroenterology 77*, 241–249.
12. Lunzer, M.A., Manning, J.A., and Ocker, R.K. (1977) *J. Biol. Chem. 252*, 5483–5487.
13. Tubbs, P.K., and Garland, P.B. (1964) *Biochem. J. 93*, 550–557.
14. Srene, P.A. (1965) *Biochim. Biophys. Acta 106*, 445–455.
15. Jacobs, R.A., and Majerus, P.W. (1973) *J. Biol. Chem. 248*, 8392–8401.
16. Halestrap, A.P., and Denton, R.M. (1974) *Biochem. J. 142*, 365–377.
17. Jolley, R.L., Cheldelin, V.H., and Newberg, R.W. (1958) *J. Biol. Chem. 233*, 1289–1294.
18. Kawaguchi, A., and Bloch, K. (1974) *J. Biol. Chem. 249*, 5793–5800.
19. Chaudhuri, D., Kushari, J., and Mukherjea, M. (1982) *Eur. J. Obstet. Gynecol. Reprod. Biol. 13*, 309–316.
20. Das, T., and Mukherjea, M. (1986) *IRCS Med. Sci. 14*, 211.
21. Laemmli, U.K., and Favre, M. (1973) *J. Mol. Biol. 80*, 575–599.
22. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497–509.
23. Ackman, R.G. (1969) in *Methods in Enzymology* (Lowenstein, J.M., ed.) Vol. 14, pp. 329–381, Academic Press, New York.
24. Ouchterlony, O. (1962) in *Progress in Allergy* (Kallos, P., and Waksman, B.H., eds.) Vol. VI, pp. 30–154, Basel, Karger.
25. Löhr, G.M., and Waller, H.D. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H.U., ed.) Vol. 2, pp. 636–643, Academic Press, New York.
26. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265–275.
27. Takahashi, K., Odani, S., and Ono, T. (1983) *Eur. J. Biochem. 136*, 589–601.
28. Robertson, A.F., and Sprecher, H. (1968) *Acta Paediatr. Scand.*, Supplement 183.
29. Basu, S.P., Rao, S.N., and Hartsuck, J.A. (1978) *Biochim. Biophys. Acta 533*, 66–73.
30. Tipping, E., and Ketterer, B. (1978) in *Transport by Proteins* (Blauser, G., and Sund, H., eds.) pp. 369–385, de Gruyter, Berlin.

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Geraniol Interferes with Membrane Functions in Strains of *Candida* and *Saccharomyces*

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Geraniol, an olefinic terpene, was found to inhibit growth of *Candida albicans* and *Saccharomyces cerevisiae* strains. Geraniol was shown to enhance the rate of potassium leakage out of whole cells and also was shown by fluorescence polarization to increase *C. albicans* membrane fluidity. Biophysical studies using differential scanning calorimetry, fluorescence polarization and osmotic swelling of phospholipid vesicles demonstrated that geraniol decreased the phase-transition temperature of dipalmitoylphosphatidylcholine vesicles, affected fluidity throughout the bilayer, particularly the central portion of the bilayers, and caused an increase in bilayer permeability to erythritol. Geraniol may have potential use as an antifungal agent.
***Lipids* 23, 534–538 (1988).**

Geraniol, an olefinic terpene isolated from rose and palm oils, is structurally similar to geranyl pyrophosphate, an essential intermediate in the ergosterol biosynthetic pathway (Fig. 1). This similarity suggested that geraniol may be an inhibitor of ergosterol biosynthetic enzymes either involved in geranyl-pyrophosphate formation (geranyl pyrophosphate synthase) or its conversion to farnesyl pyrophosphate (farnesyl pyrophosphate synthase). Many antifungals such as the azoles (econazole, ketoconazole, miconazole) interfere with ergosterol biosynthesis by inhibiting specific steps such as lanosterol demethylation (1,2) or, as in the case of naftifine, inhibit squalene epoxidase (3). Other antifungals such as the azasterols inhibit sterol methylation (4,5). Another category of antifungals, the polyenes, inhibit fungal cells having sterols in their cell membrane. Cell lines in *Candida* and *Saccharomyces* resistant to the polyene antibiotics generally have ergosterol intermediates rather than ergosterol in their cell membranes (6,7).

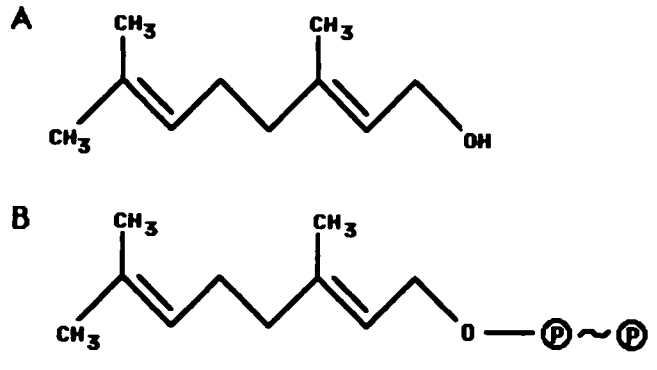

FIG. 1. Structural comparison of (A) geraniol and (B) geranyl pyrophosphate.

In this report, we demonstrate that three clinical isolates of *Candida albicans* and one haploid *Saccharomyces cerevisiae* lab strain are sensitive to the effects of geraniol and that geraniol causes changes in membrane dynamics rather than inhibiting ergosterol biosynthesis. In ergosterol biosynthesis, geranyl pyrophosphate is converted to farnesyl pyrophosphate, and the latter differs from the former in containing an additional isoprenoid unit. Attempts to inhibit growth of several *Saccharomyces* strains using varying concentrations of farnesol (exceeding 5-fold the concentration of geraniol that totally inhibited growth) resulted in no growth inhibition. This suggests that geraniol inhibition is not merely due to the hydrophobicity of the geraniol molecule. Here we compare the effect of geraniol on membrane properties of living cells with its effects on well-defined phospholipid bilayers.

MATERIALS AND METHODS

Yeast strains and media. The three *C. albicans* isolates were A26 (a clinical isolate received from R. Gordee, Eli Lilly & Co., Indianapolis, IN), hOG 357 (an arginine, adenine, methionine auxotrophic strain received from P. Magee, Michigan State University) and ATCC strain 44829 (a clinical isolate mutagenized and selected for adenine auxotrophy). The wild-type, A184D, *S. cerevisiae* strain has been described (8). Yeast cells were grown in a yeast-complete medium (YPD) containing 1% yeast extract, 2% Bacto-peptone, 2% dextrose and 2% agar. Ergosterol was added at 5×10^{-5} M to complete medium from a stock solution of 0.2% ergosterol in Tween 80/ethanol (1:1, v/v). Geraniol was added from a stock solution of geraniol/ethanol (1:1, v/v).

Liposome vesicle preparation and differential scanning calorimetry. Multilamellar vesicles (MLV) comprised of dipalmitoylphosphatidylcholine (DPPC) were made containing between 0 and 20 membrane mol% geraniol by the method of Bangham (9) at 65 C in 100 mM potassium phosphate buffer, pH 7.5, and processed in a "Liposor" lipid vesicle maker (Lidex Technologies, Bedford, MA). The lipid concentration was 100 mg/ml and the weight of samples ca. 6.5 mg. Phase transitions were measured in a Perkin-Elmer Series 7 Differential Scanning Calorimeter.

Fluorescence polarization studies. Fluorescence polarization measurements were made on both DPPC lipid vesicles as well as *C. albicans* whole cells.

Lipid vesicles. MLV were made from DPPC as described above at 10 mg/ml and were sonicated in 20 mM potassium phosphate, pH 7.5, buffer on a Heat Systems W220-F cell disrupter for five min until the solution cleared. The sonicated vesicles were either 100 mol% DPPC or 80 mol% DPPC/20 mol% geraniol. 9-Anthroyloxy-stearic acids labeled at positions 2, 6, 9 or 12 were incorporated into the bilayers during their synthesis at

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Abbreviations: AS, anthracene stearic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; MIC, minimum inhibitory concentration; MLV, multilamellar vesicles; P, polarization; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; YPD, yeast-complete medium.

a ratio of 200 lipids/AS. Polarization measurements were made on a Perkin-Elmer MPF-66 Fluorescence Spectrophotometer at decreasing temperatures between 50 C and 30 C. Excitation was at 361 nm and emission at 446 nm. Temperature in the cuvettes was monitored accurately by a thermoprobe. Polarization values were calculated as described by Chefurka et al. (10) by the equation:

$$P = \frac{I_{vv} - G\,(I_{vh})}{I_{vv} + G\,(I_{vh})}$$

where I represents fluorescent intensity, G is the "grating factor" (I_{hv}/I_{hh}), and h and v refer to the position of the polarizers either horizontal or vertical.

Whole cells. C. albicans strain, hOG 357, was grown overnight to mid-log phase (O.D.$_{660}$ 0.5) in YPD medium, pelleted and washed two times in a 10 mM Tris, pH 7.0, 0.9% NaCl buffer. Cells were resuspended in the same buffer also containing 1% Tween 80 at a final concentration of 4×10^6 cells/ml. The presence of Tween 80, a non-ionic polyoxyethylene detergent containing oleic acid, enhanced geraniol solubility and was included at the same concentration for all cell suspensions. Membrane polarization was determined for the cell membrane interior by 1,6-diphenyl-1,3,5-hexatriene (DPH) and for the aqueous-membrane interface by 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH). Fluorescent labels (43 μM) were dispersed into cell populations using N,N-dimethylformamide as carrier. Cells were preincubated at 37 C for one hr, after which various concentration of geraniol were added. Cells containing fluorescent label and geraniol were further incubated for 90 min. Wavelengths for excitation were 351 nm (DPH) and 358 nm (TMA-DPH) and emission 430 nm (DPH) and 426 nm (TMA-DPH) with experimental temperatures set at 37 C. Polarization (P) values are the average of five separate determinations. All fluorescent labels were purchases from Molecular Probes (Eugene, OR).

Erythritol permeability studies. Egg phosphatidylcholine lipid vesicles containing varying amounts of geraniol (from 0 to 20 membrane mol%) were made by the same method used in the differential scanning calorimetry experiments but at a concentration of 10 mg/ml. The vesicles were rapidly injected into a swelling buffer of 10 mM potassium phosphate and 40 mM erythritol, pH 7.0. Over the time of the measurement (ca. 30 sec), glucose does not cross the bilayer; however, the smaller erythritol molecule does cross down its concentration gradient into the vesicles, causing a hypotonic state. The subsequent water movement results in vesicle swelling, which is measured as a change in absorbance. Lipid vesicles are known to behave as almost perfect osmometers (11), so initial swelling rates (12,13) of the vesicles are an accurate indication of erythritol permeability. Absorbances at 500 nm were recorded in a temperature controlled Beckman DU-8 Computing Spectrophotometer. Results are expressed as initial swelling velocity dl/A/dt% and reported as the average of four to six determinations.

Potassium leakage studies. C. albicans strain, hOG 357, was grown, harvested and resuspended in buffer essentially as described for fluorescence polarization using whole cells except that cells were resuspended at a concentration of 3×10^7 cells/ml. Potassium leakage was measured with a K^+ specific electrode (Microelectrodes, Londonderry, NH) on an Orion 901 Microprocessor Ionanalyzer. After six hr, cells were completely lysed in 3% Triton X-100, and the maximum sequestered K^+ was determined.

RESULTS AND DISCUSSION

Minimal inhibitory concentration (MIC) of geraniol. Yeast cells grown overnight in a YPD (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) were plated onto YPD plates containing varying concentrations of geraniol at a density of 10^5 cells per plate. Plates were scored after 48 hr, and the results are presented in Table 1. The minimum inhibitory concentration (MIC) of geraniol is defined as that concentration that completely prevents growth after 48 hr. All three *Candida* strains as well as the *Saccharomyces* strain were approximately equally sensitive to geraniol. The numbers in parentheses indicate the MIC in mM concentrations. To determine whether ergosterol supplementation reverses the antifungal effect, plates containing 5×10^{-5} M ergosterol and geraniol were tested. The results with ergosterol supplementation were the same as without ergosterol supplementation, indicating that inhibition of ergosterol synthesis was not the mode of geraniol inhibition or that ergosterol was not taken up from the growth medium. Because liquid YPD cultures innoculated to a visible turbid density when incubated overnight with inhibitory concentrations of geraniol often cleared, this suggested that geraniol may act at the level of the plasma membrane, causing cell lysis.

Lipid vesicle fluidity. To ascertain whether geraniol affects cell membranes by altering bilayers properties, geraniol was incorporated into phospholipid vesicles. Differential scanning calorimetry indicated geraniol broadened and decreased the phospholipid phase transition temperature of the synthetic phospholipid DPPC. Figure 2 demonstrates that increasing concentrations of geraniol from 0 to 20 mol% broadened and decreased the phase transition temperatures of the liposome vesicles 2.2 C indicating that geraniol disorders the bilayers. This suggests geraniol also may have a fluidizing effect on yeast cell membranes.

To test membrane fluidity, fluorescence polarization was employed (14). Anthracene-labeled stearic acids (AS)

TABLE 1

Minimal Inhibitory Concentrations (MIC) of Geraniol for *Candida* and *Saccharomyces* Strains

Strain	MIC (μg/ml)[a]
Candida albicans	
A26	309 (2)[b]
hOG 357	309 (2)
ATCC 44829	463 (3)
Saccharomyces cerevisiae	
A184D	463 (3)

[a]All inoculums were 10^5 cells/plate. Results represent data from at least three independent experiments for each strain.

[b]Number in parentheses indicates MIC of geraniol in mM concentrations.

have been used to probe the effect of a number of compounds on fluidity as a function of depth in the bilayer (10). Fluorescence polarization measurements also can be used to determine the phase transition temperature of bilayers comprised of synthetic phospholipids (10,15).

The results in Figure 3 show that, as expected, polarization values decreased (relative fluidity increased) from position 2 near the membrane surface to position 12 near the bilayer interior. For all four positions and in the gel and liquid crystalline states, geraniol decreased the P-values (increased fluidity). For DPPC in the liquid crystalline state, the average decrease in P caused by geraniol over the temperature range 50 C to 41 C can be used to compare quantitatively the effect of geraniol at different depths in the bilayer. The values expressed as $\Delta P_{average}$ for the 10 temperatures are: 2 AS, 0.0023; 6 AS, 0.0067; 9 AS, 0.0038; and 12 AS, 0.0041. Clearly, geraniol enhances fluidity at all positions along the chain with the largest effect associated with position 6. This agrees with the structure of geraniol (Fig. 1), which has methyl branches at positions 3 and 7 and so would be expected to disorder bilayers in the central part of the chain more than at either end. These results are in contrast to those for n-alkanols, which mostly perturb the bilayer center (16). In agreement with the differential scanning calorimetry measurements reported in Figure 2, 20 mol% geraniol broadens and decreases the phase transition temperature of DPPC by about 2 C.

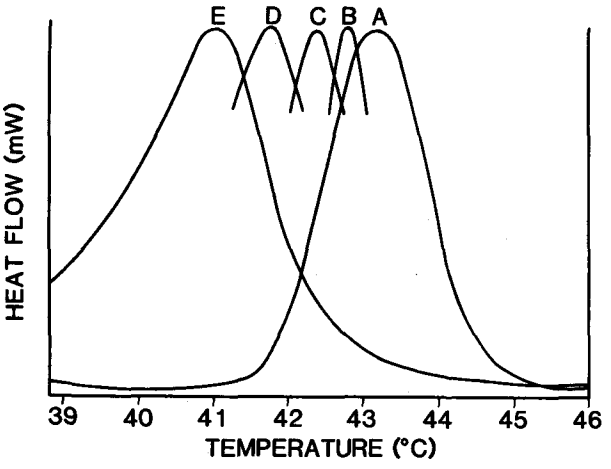


FIG. 2. Effect of geraniol on the phase transition of dipalmitoylphosphatidylcholine vesicles. Geraniol membrane concentration: (A) 0 mol%, (B) 5 mol%, (C) 10 mol%, (D) 15 mol% and (E) 20 mol%.

Cell fluidity. Fluidity also was ascertained by fluorescence polarization with *C. albicans* whole cells. The bilayer interior probe DPH indicated an enhanced membrane fluidity as a function of geraniol concentration (Fig. 4A), while the surface probe TMA-DPH also showed that geraniol enhanced probe motion (fluidity) at the aqueous interface (Fig. 4B). These results confirm those with DPPC lipid vesciles (Fig. 3) and show that geraniol's

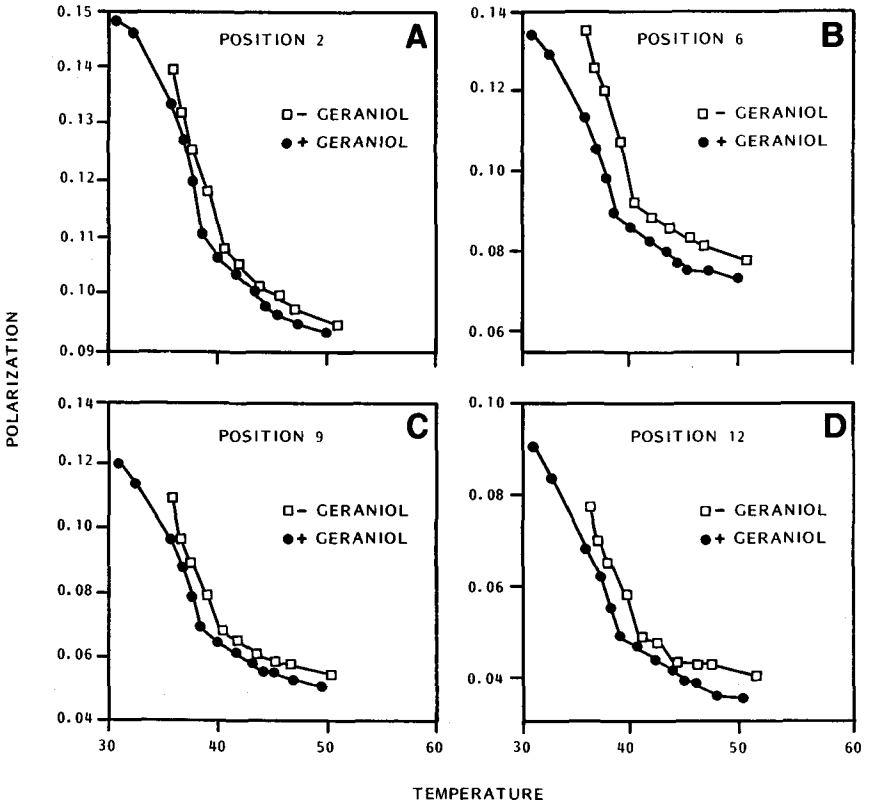
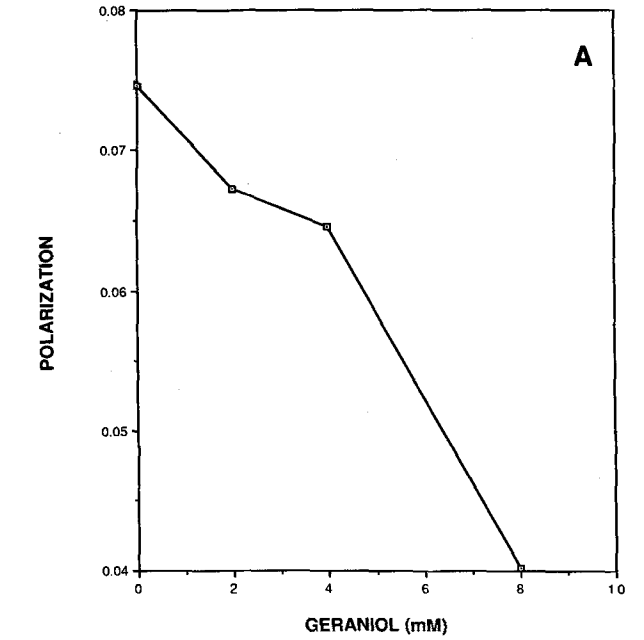


FIG. 3. Effects of geraniol on the polarization of DPPC vesicles as a function of temperature. Geraniol concentration is 20 mol% with respect to DPPC. Fluorescent labels are 2 AS (panel A), 6 AS (panel B), 9 AS (panel C) and 12 AS (panel D). Results, expressed as P, are the average of five determinations and vary 0.0015.

ability to increase bilayer fluidity extends to natural membranes.

Lipid vesicle permeability. A light-scattering technique measuring the permeability of erythritol was used to quantitate the effect of geraniol on general membrane permeability. Geraniol-induced erythritol permeability at different temperatures is shown in Figure 5. Geraniol concentrations are expressed as the mol% geraniol/phosphatidylcholine. Concentrations range from 0% (curve A) to 20 mol% geraniol (curve F). These results also demonstrate that geraniol enhancement of permeability is greater in the more fluid bilayer, that is, at higher temperatures.



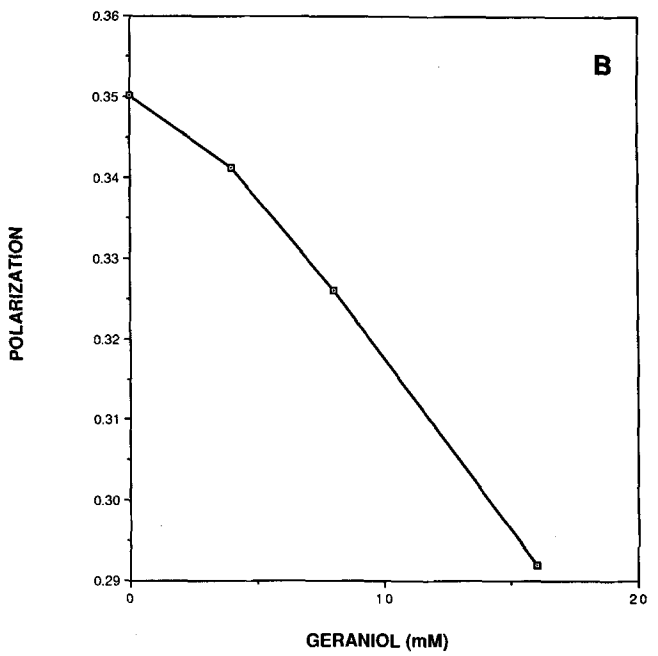


FIG. 4. Effect of geraniol on the polarization of *C. albicans* whole cell membranes. Panel (A) DPH; Panel (B) TMA-DPH. Concentrations of geraniol varied from 0–16 mM.

Potassium leakage from whole cells. The observations that geraniol alters phase transition temperature of liposome vesicles, increases bilayer fluidity and increases liposome permeability to erythritol suggested that the antifungal activity of the molecule also may enhance the rate of solute leakage (K^+) out of whole cells. Results (Fig. 6) are expressed as the percentage of internal sequestered K^+ leaking out of the cell as a function of time. These results indicate that increasing geraniol concentrations do enhance K^+ leakage most markedly in the first two hr. For example, after two hr cells incubated without geraniol showed a 5% K^+ leakage, whereas cells incubated with 12 mM geraniol showed a 28% K^+ leakage.

In conclusion, the experiments presented here on *Candida* and *Saccharomyces* fungal strains, as well as on protein-free phospholipid vesicles, may help to understand the mode of action of geraniol in preventing fungal

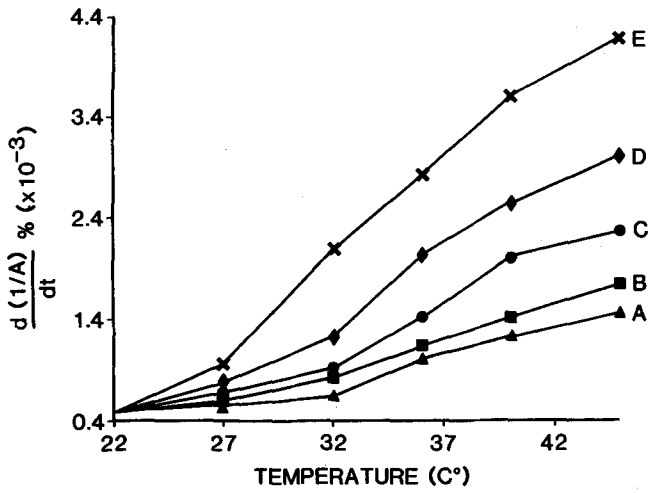


FIG. 5. Effect of geraniol on the permeability of egg phosphatidylcholine vesicles to erythritol. Geraniol membrane concentration: (A) 0 mol%; (B) 5 mol%; (C) 10 mol%; (D) 15 mol%; and (E) 20 mol%.

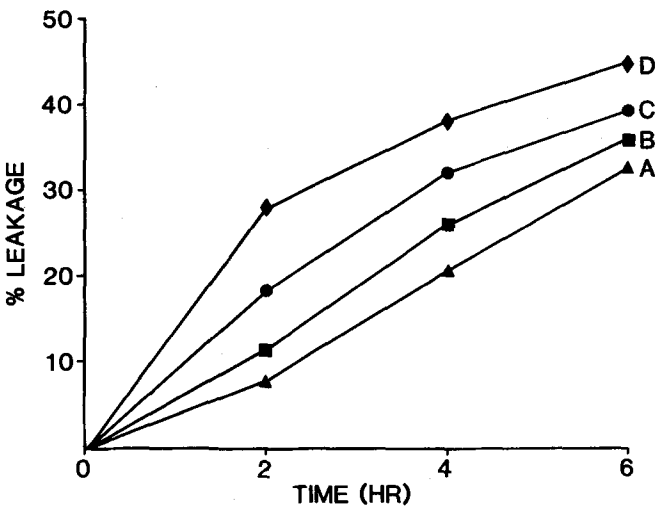


FIG. 6. Effect of geraniol on the leakage of K^+ from *C. albicans* strain hOG 357. Results are expressed as the percentage of initially sequestered K^+ leaking out with time. Geraniol concentration in the bathing solution (10 mM Tris, pH 7.0, 1% Tween 80, 0.9% NaCl). (A) 0 mM; (B) 4 mM; (C) 8 mM; and (D) 12 mM.

growth. Geraniol does not appear to inhibit ergosterol biosynthesis as its primary mode of action but instead appears to partition into membranes where it alters bilayer properties. Upon incorporation of geraniol, membranes become more fluid (as indicated by a broadening and decrease in the phase transition temperature of DPPC vesicles as well as by polarization measurements with AS labels with lipid vesicles and DPH and TMA-DPH measurements on whole cells), while general bilayer permeability is increased (as indicated by vesicle swelling in isomolar erythritol and K^+ leakage from cells).

Cushley et al. (17) have reported similar membrane effects caused by the larger brancher chain alcohol, phytol. This alcohol also broadened and decreased phospholipid phase transitions, decreased membrane order and increased permeability to the cationic NMR shift reagent Pr^{3+}. It was proposed that phytol acts as a membrane-destabilizing agent, increasing membrane permeability. In light of these studies, it is not surprising that geraniol also destabilizes membranes.

Inhibition of cell growth may be the result of altered membranes with the symptomatic loss of cellular potassium. We conclude that the antifungal activity of geraniol resides with its ability to disrupt membrane bilayer structures and, therefore, cellular processes.

The experiments presented here represent an attempt to link biophysical data with biological processes to establish a mode of action of any drug that is thought to be membrane-disruptive. The assessment of geraniol as an antifungal agent for plants or animals remains to be determined.

ACKNOWLEDGMENTS

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REFERENCES

1. Borgers, M. (1980) *Rev. Infect. Dis. 2,* 200-210.
2. Shepherd, M.G., Poulter, R.T.M., and Sullivan, P.A. (1985) *Ann. Rev. Microbiol. 39,* 579-614.
3. Poltauf, F., Daum, G., Zunder, G., Högenauer, G. Schulz, G., and Seidl, G. (1982) *Biochim. Biophys. Acta 712,* 268-273.
4. Oehlschlager, A.C., Angus, R.H., Pierce, A.M., Pierce, H.D. Jr., and Srinivasan, R. (1984) *Biochemistry 23,* 3582-3589.
5. Pierce, H.D. Jr., Pierce, A.M., Srinivasan, R., Unrau, A.M., and Oehschlager, A.C. (1978) *Biochim. Biophys. Acta 529,* 429-437.
6. Henry, S.A. (1982) in *The Molecular Biology of the Yeast* Saccharomyces, *Metabolism and Gene Expression* (Strathern, J.N., Jones, E.W., and Broach, J.R., eds.) pp. 101-158, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
7. Pierce, A.M., Pierce, H.D. Jr., Unrau, A.M., and Oehlschlager, A.C. (1978) *Can. J. Biochem. 56,* 135-142.
8. Molzahn, S.W., and Woods, R.A. (1972) *J. Gen. Microbiol. 72,* 339-348.
9. Bangham, A.D. (1968) *Progr. Biophys. Mol. Biol. 18,* 29-95.
10. Chefurka, W., Chatelier, R.C., and Sawyer, W.H. (1987) *Biochim. Biophys. Acta 896,* 181-186.
11. Bangham, A.D., DeGier, J., and Greville, G.D. (1967) *Osmotic Chem. Phys. Lipids 1,* 225-246.
12. Blok, M.L., van Deenan, L.L.M., and DeGier, J. (1976) *Biochim. Biophys. Acta 433,* 1-12.
13. DeGier, J., Mandersloot, J.G., and van Deenen, L.L.M. (1986) *Biochim. Biophys. Acta 150,* 666-675.
14. Schacter, D. (1984) *Hepatology 4,* 140-151.
15. Stelzer, R.J., and Gordon, M.A. (1985) *Biochim. Biophys. Acta 812,* 361-368.
16. Zavoico, G.B., Chandler, C., and Kutchai, H. (1985) *Biochim. Biophys. Acta 812,* 299-312.
17. Cushley, R.J., Forrest, B.J., Gillis, A. and Tribe, J. (1979) *Can. J. Chem. 57,* 458-465.

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Polyunsaturated Fatty Acids of Serum Lipids in Myocardial Infarction

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Two groups of volunteers had blood drawn for serum analysis of fatty acids. The first group was comprised of patients admitted to the hospital with possible myocardial infarction (MI). Blood was drawn at admission and at 12, 24 and 48 hr. These patients were subsequently divided into three groups, those with MI, those without (No MI) and those taking prostaglandin inhibitors (PGI), on the basis of the cardiac enzymes, electrocardiograms and clinical history. A fourth group of Normal non-stressed people was also drawn at 0, 12, 24 and 48 hr for comparison. Fatty acid composition of phospholipids (PL), nonesterified fatty acids (NEFA), triglycerides (TG) and cholesteryl esters (CE) was determined by capillary gas chromatography (GC), and comparisons were made between the MI, No MI, PGI and Normal groups. Total NEFA were significantly elevated in patients admitted for possible MI compared with Normals. Those patients with MI had marginally higher levels of NEFA than the No MI group at each sampling time, but this difference was not statistically significant. The MI, No MI and PGI groups had significantly different fatty acid patterns in NEFA with reduced percentages of arachidonic acid (AA) than controls. The fatty acid patterns in the four lipid classes showed few significant differences comparing the MI, No MI and PGI groups. The regular use of prostaglandin inhibitors before hospitalization for chest pain was associated with a reduced frequency of MI ($p < 0.002$). NEFA levels, nonesterified AA levels and fatty acid patterns in this group did not differ from those patients not taking prostaglandin inhibitors.

Lipids 23, 539-545 (1988).

Myocardial infarction (MI) usually occurs as the result of partial or complete occlusion of one or more coronary arteries. Arachidonic acid (AA, 20:4ω6) metabolism may play a role in this pathology by several mechanisms: vasospasm mediated by thromboxane A_2 (TXA_2) production and prostacyclin (PGI_2) inhibition, platelet clot formation stimulated by TXA_2 due to vascular injury or plaque formation, and vascular occlusion by atherosclerotic plaque caused by vascular endothelial cell injury.

The general elevation of nonesterified fatty acids (NEFA) seen in association with myocardial damage may cause an increase in myocardial oxygen consumption, increased incidence of arrhythmias, and increased size of the infarction (1-4). Elevated NEFA have been shown to stimulate platelet aggregation (5). Thus, elevations of NEFA may contribute to the progression of tissue injury in MI by both increasing tissue oxygen requirements and by promoting vascular occlusion (Fig. 1). Two methods used to decrease the death rate in patients with MI have had some success: the inhibition of TXA_2 production and platelet activation using low-dose aspirin (6,7), and the suppression of circulating NEFA using glucose-insulin-potassium infusions (8).

The following study, which was approved by the University of Minnesota Committee on Human Investigations, sought to clarify the effects of stress and pain on elevation of NEFA with and without MI. Quantities of nonesterified AA in NEFA and fatty acid patterns in NEFA, cholesteryl esters (CE), triglycerides (TG) and phospholipids (PL) were compared in three types of patients: those with symptoms of MI and medically proven MI, those with symptoms of MI, but with no MI, and Normal controls with no symptoms of MI.

METHODS

A group of volunteers had blood drawn for serum analysis of fatty acids. The group was comprised of patients admitted acutely to a small, rural hospital for chest pain with possible MI. Blood was drawn on admission, at 12 hr, at 24 hr and at 48 hr for cardiac enzyme analysis and fatty acid analysis. Timing of each sample depended on admission and, therefore, was random with regard to prandial status. Patients were offered a no-added salt, low-cholesterol diet, either full liquids or solid food, but oral intake varied widely depending on appetite and clinical circumstances from 0 to 2,500 calories per 24 hr. Approximately 30% of caloric intake in either diet was from lipids, with equal amounts of saturated and unsaturated fats. In addition, IV access was maintained with 5% glucose solution until a definitive diagnosis of MI could be excluded and was continued at least 48 hr if MI was found. Intake of IV glucose by this route could vary from 240 to 600 calories per 24 hr. Subsequent to the completion of the fatty acid analyses, this group of patients was sorted into two groups: one in which MI had been confirmed by enzyme studies and electrocardiograms (experimental group = MI), and one that had not experienced MI on the basis of cardiac enzymes (control group = No MI).

A third group was made up of normal, non-stressed health care providers without chest pain and without MI (Normal). The Normal group (n = 10) had blood drawn at four times: 0, 12, 24 and 48 hr. The first and fourth samples were drawn after an overnight fast. The second and the third samples were taken two to three hr postprandial.

Pertinent clinical history for each group was obtained, and special attention was given to medications known to inhibit prostaglandin synthesis. Whenever possible, the use of these drugs was confirmed by direct questioning of the patient or review of outpatient clinic records.

Lipid extraction, methylation and gas chromatography. Serum samples were kept frozen until analysis.

[1]Part of this work was presented at the 78th American Oil Chemists' Society Annual Meeting, May 17-21, 1987.

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Abbreviations: AA, arachidonic acid; CE, cholesteryl esters; DHLA, dihomogamma linolenic acid (20:3ω6); FAME, fatty acid methyl esters; MI, myocardial infarction; NEFA, nonesterified fatty acids; PGI, prostaglandin inhibitor; PGI_2, prostacyclin; PL, phospholipids; PUFA, polyunsaturated fatty acids; TG, triglycerides; ANOVA, analysis of variance; GC, capillary gas chromatography.

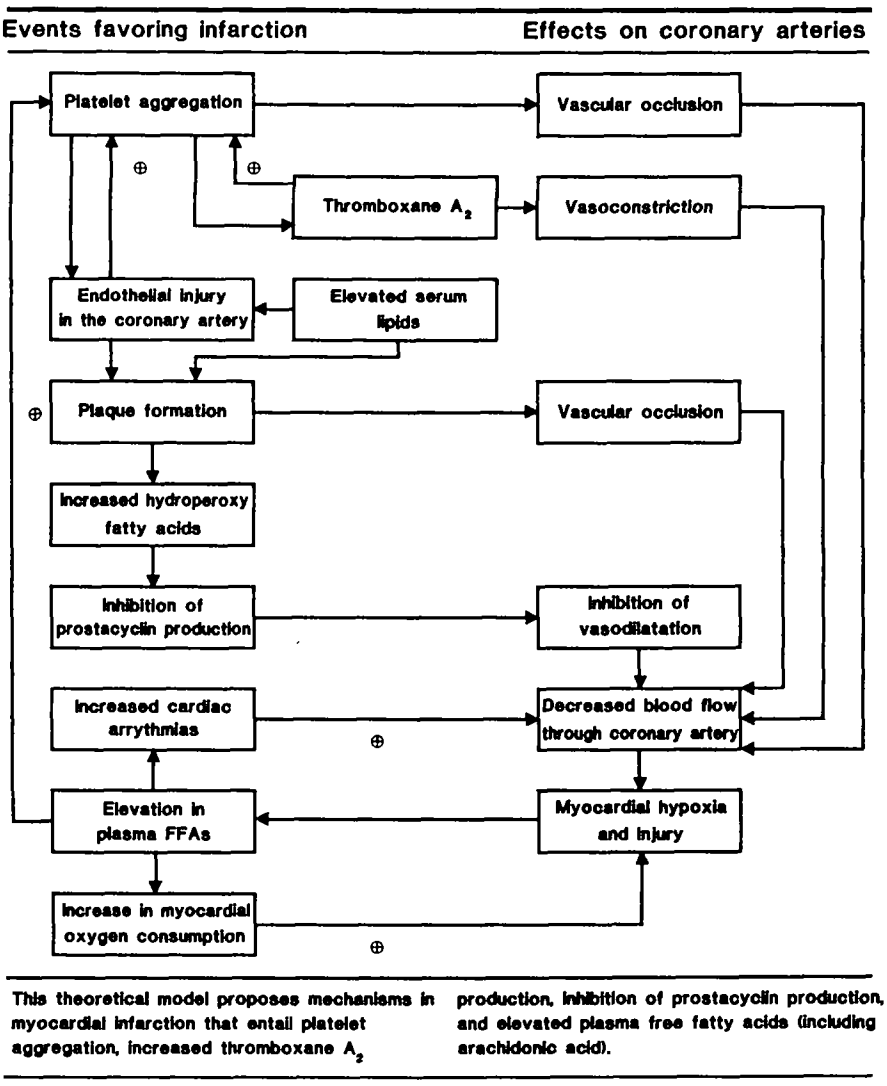

FIG. 1. Proposed mechanism of changes in myocardial infarction. This theoretical model proposes mechanisms in myocardial infarction that entail platelet aggregation, increased TXA_2 production, inhibition of PGI_2, and elevated plasma NEFA (including AA) (11). ⊕ Symbolizes positive-feedback routes.

Heptadecanoic acid (17:0) was added to each sample as internal standard. Lipids from each 2-ml sample were extracted with chloroform/methanol (2:1, v/v). The samples were mixed well and centrifuged. The aqueous layer was drawn off and the chloroform layer filtered to remove the protein. This layer then was dried under a gentle stream of nitrogen and redissolved in 100 μl of chloroform.

The lipid extracts were spotted onto 20 × 20 cm sheets of silicic acid-impregnated ITLC paper (Gelman Sciences, Inc.) and developed in 30–60 C petroleum ether/diethyl ether/acetic acid (90:10:1). The chromatograms were sprayed with 0.1% 2′,7′-dichlorofluorescein solution and illuminated with UV light. The individual lipid classes appeared as distinct bands and were put into glass tubes with teflon-lined caps. These were transesterified with 2 ml of 14% boron trifluoride/methanol (w/v) at 85 C for 1½ hr for PL and CE, and ½ hr for FA and TG. After esterification, the fatty acid methyl esters (FAME) were extracted with petroleum ether (30–60 C), the samples were taken to dryness under nitrogen and redissolved in a minimum of petroleum ether for capillary gas chromatography (GC) analysis.

GC analysis. GC analysis was carried out on a 50 m × 0.2 mm fused silica capillary column coated with FFAP (Scientific Glass Engineering, Austin, TX). The column was temperature-programmed from 190–220 C at 2 C/min, with a final hold of 45 min. The carrier gas was helium, at a flow rate of about 0.68 ml/min. The split ratio was 1:99. Identification of FAME was made by comparison with authentic standards. Peak areas were calculated by a microprocessor. Data were treated by analysis of variance (ANOVA).

RESULTS

Of 58 patients admitted with possible MI, 14 had enzyme-confirmed MI, and 44 did not (No MI). Of these patients, serum samples for lipid analysis were available from 11

TABLE 1

Total Nonesterified Fatty Acids in Normal Individuals and in Patients with and without Myocardial Infarction

Time	Normal	No MI	MI	PGI	P-value[a]		
					No MI	MI	PGI
Admission	7.30 ± 1.28 (n = 9)	14.55 ± 1.56 (n = 16)	23.56 ± 6.07 (n = 7)	17.12 ± 2.79 (n = 10)	<0.01	<0.01	<0.01
12 hr	5.33 ± 0.67 (n = 9)	11.21 ± 2.18 (n = 10)	21.47 ± 2.15 (n = 5)	11.67 ± 2.83 (n = 8)	<0.05	<0.001	<0.05
24 hr	5.19 ± 0.74 (n = 8)	17.59 ± 2.96 (n = 10)	23.93 ± 5.05 (n = 4)	15.01 ± 3.13 (n = 7)	<0.01	<0.001	<0.01
48 hr	6.21 ± 1.21 (n = 9)	15.13 ± 0.62 (n = 5)	21.45 ± 4.29 (n = 3)	10.46 ± 2.61 (n = 3)	<0.001	<0.001	NS

Values are mean ± SEM (mg/dl).

[a]p-Value vs Normal, ANOVA.

TABLE 2

Total Nonesterified Arachidonic Acid in Normal Controls and in Patients with and without Myocardial Infarction

Time	Normal	No MI	MI	PGI
Admission	0.275 ± 0.060 (n = 9)	0.241 ± 0.038 (n = 16)	0.247 ± 0.057 (n = 7)	0.210 ± 0.045 (n = 10)
12 hr	0.212 ± 0.035 (n = 9)	0.209 ± 0.027 (n = 10)	0.213 ± 0.054 (n = 5)	0.137 ± 0.025 (n = 8)
24 hr	0.264 ± 0.029 (n = 8)	0.165 ± 0.033* (n = 10)	0.409 ± 0.090 (n = 4)	0.343 ± 0.089 (n = 5)
48 hr	0.173 ± 0.038 (n = 9)	0.242 ± 0.026 (n = 5)	0.357 ± 0.130 (n = 3)	0.290 ± 0.088 (n = 3)

Values are mean ± SEM (mg/dl).

*$p < 0.05$ compared with Normal and PGI; $p < 0.01$ compared with MI.

patients with MI and 35 patients with No MI. Subsequent to the analyses, it was determined that a subgroup of the No MI group had been treated with anti-inflammatory drugs (n = 19). From this prostaglandin inhibitor (PGI) subgroup, 13 sets of serum samples were available for analysis. None of the MI patients were on chronic antiprostaglandin medications. In some cases, it was impossible to obtain all four serum samples from each patient, or some samples were too small to analyze. These variations in group size are reflected in the summary tables.

Total NEFA measured for the four groups are shown in Table 1. ANOVA showed significant differences among the four groups, Normal, No MI, MI and PGI, at each time of blood sampling. The No MI and MI groups had significantly higher total NEFA than the Normal group at each time period. NEFA levels in the PGI group did not show sustained elevation at 48 hr in contrast with the No MI and MI groups.

The No MI group had significantly lower NEFA than the MI group at 12 hr only ($p < 0.005$, Student's t-test). Linear regression analyses were done of the No MI group and the MI group (total NEFA vs time). For the No MI group, R = −0.032 (95% confidence interval for R = 0.207 to −0.268); and for the MI group, R = −0.048 (95% confidence interval for R = 0.412 to −0.487). These results suggest that total NEFA is independent of time in the No MI and MI groups during the 48-hr interval studied.

Serum AA levels of the NEFA were calculated for each of the study groups (Table 2). There were no significant differences in mean quantities of AA among the groups except that the No MI group had a significant decrease at 24 hr after admission compared with the MI group ($p < 0.01$) and to the Normal and PGI groups ($p < 0.05$) (Table 2). The percentage of NEFA made up by AA was significantly greater in the control samples than in the MI and No MI groups at admission and at 24 hr ($p < 0.01$) (Table 3). The only difference between the MI and No MI groups occurred at 12 hr after admission when the percentage AA was significantly lower in the MI group ($p < 0.05$). Percentage of AA was significantly greater in the PGI group compared with the No MI group at 24 hr only ($p < 0.05$).

Table 4 shows the fatty acid pattern in NEFA for initial fasting samples of controls and the differences from the patterns in the MI, No MI and PGI groups. 16:1ω7 was significantly increased in the MI, No MI and PGI

TABLE 3

Percentage of Nonesterified Fatty Acids Made up of Arachidonic Acid in Controls and in Patients With and Without Myocardial Infarction

Time	Normal	No MI	MI	PGI
Admission	3.660 ± 0.617* (N = 9)	1.673 ± 0.205 (n = 16)	1.260 ± 0.408 (n = 7)	1.415 ± 0.262 (n = 10)
12 hr	4.323 ± 0.991 (n = 9)	2.306 ± 0.389 (n = 10)	0.988 ± 0.262** (n = 5)	1.481 ± 0.307 (n = 8)
24 hr	5.505 ± 0.561* (n = 8)	1.348 ± 0.388 (n = 10)	1.715 ± 0.259 (n = 4)	5.490 ± 1.95** (n = 6)
48 hr	3.254 ± 0.871 (n = 9)	1.614 ± 0.199 (n = 5)	1.923 ± 0.942 (n = 3)	3.280 ± 1.44 (n = 3)

Values are mean ± SEM in % of total NEFA.

*$p < 0.01$ compared with the No MI and the MI groups; **$p < 0.05$ compared with the No MI group.

TABLE 4

Fatty Acid Percentages in NEFA for Initial Fasting Control Subjects and Significances of Comparisons

Fatty acid	Normal	No MI	MI	PGI
14:0	1.27 ± 0.47	$p < 0.01$	NS	NS
16:0	25.99 ± 2.67	NS	NS	NS
16:1ω7	2.24 ± 0.52	$p < 0.001$	$p < 0.05$	$p < 0.05$
18:0	18.24 ± 2.90	$p < 0.05$	$p < 0.05$	NS
18:1ω9	26.31 ± 3.08	NS	$p < 0.05$	NS
18:2ω6	15.20 ± 1.61	NS	NS	NS
20:3ω6	0.24 ± 0.14	NS	NS	NS
20:4ω6	3.66 ± 0.65	$p < 0.001$	$p < 0.01$	$p < 0.01$
22:6ω3	0.11 ± 0.11	NS	NS	NS

Values are mean ± SEM in % of total NEFA.

groups. 18:0 was significantly decreased in both the MI and No MI groups. 18:1ω9 was significantly increased in the MI group only. 20:4ω6 was significantly decreased in the MI, No MI and PGI groups. Other individual fatty acids were not significantly different.

Table 5 compares the fatty acid patterns for admission samples in the MI, No MI and PGI groups for each of the major serum lipid classes. For the No MI group compared with the MI group, only 16:1ω7 was significantly elevated in PL, and 14:0 was significantly elevated in NEFA. For the PGI group compared with the MI group, 22:6ω3 was significantly elevated in PL, 18:0 was elevated in NEFA, 18:2ω6 and 20:3ω6 (DHLA, dihomogamma linolenic acid) decreased in NEFA, 18:2ω6 decreased in TG, and 16:0 decreased in CE. Only 22:6ω3 was significantly increased in PL of the PGI group compared with the No MI group. There were no significant differences in the major fatty acids (16:0, 18:0, 18:1ω9, or 18:2ω6) between the MI and No MI groups in any of the four lipid classes. The prostaglandin precursors, 20:3ω6 and 20:4ω6, also were not significantly different in the four lipid classes between the three groups, except 20:3ω6 in NEFA for the PGI group.

None of 19 patients regularly taking prostaglandin inhibitors proved to have MI. Therefore, we compared the MI group and all patients not suffering a heart attack (No MI and PGI groups) with respect to the 10 clinical variables that are listed in Table 6. *The regular use of prostaglandin inhibitors before hospitalization for chest pain was associated with an outcome of No MI to a high level of statistical significance ($p < 0.002$).* The only other variable showing a difference between the two groups was cardiac arrhythmia, which occurred during the course of hospitalization more frequently in the MI group. This difference also was statistically significant. The highest diastolic blood pressure measurement recorded during the hospitalization showed an elevation in the MI group compared with the No MI group, which approaches statistical significance.

In addition, a multivariate analysis was performed to examine the confounding effect of the various covariates on the use of prostaglandin inhibitors in MI. Stepwise logistic regression was used to assess the ability of prostaglandin inhibitors to predict MI in the presence of the nine covariates. The likelihood ratio chi-square results from this stepwise logistic regression analysis indicated that non-use of prostaglandin inhibitors is a statistically significant predictor of MI in the presence of the covariates ($p < 0.01$).

DISCUSSION

This study confirms previous findings that NEFA are elevated in association with MI (1–4). The fact that elevated NEFA are seen in patients with symptoms of MI but without evidence for myocardial damage suggests that this lipolysis probably is mediated through the sympathetic nervous system and is associated with stress. Elevations in NEFA are related to catecholamine elevations in patients with MI (9). The lipolysis caused by catecholamine mobilizes NEFA from adipocyte TG, which are low in AA. This could explain the decreased percentage of AA in the No MI and MI groups. The AA concentration did not change, although the total NEFA increased. Work by McDaniel et al. has shown similar NEFA patterns in patients with MI. When total NEFA values were suppressed by glucose-insulin-potassium infusion, the relative percentage of AA increased (10).

TABLE 5

Fatty Acid Percentages of the Major Lipid Classes on Admission for Patients with and without Myocardial Infarction

	PL			TG			NEFA			CE		
Fatty acid	No MI (n = 20)	MI (n = 11)[a]	PGI (n = 13)	No MI (n = 19)	MI (n = 11)	PGI (n = 13)	No MI (n = 17)	MI (n = 7)	PGI (n = 9)	No MI (n = 20)	MI (n = 10)	PGI (n = 13)
14:0	0.35±0.02	0.31±0.04	0.35±0.04	2.11±0.22	1.51±0.15	1.76±0.35	2.94±0.34[b]	1.44±0.35	1.98±0.45	0.68±0.05	0.79±0.12	0.55±0.09
16:0	26.26±0.51	25.78±0.72	28.25±0.79	25.93±0.84	24.29±1.17	24.85±0.75	26.13±1.37	22.06±2.36	24.81±1.57	11.88±0.40	12.69±0.62	11.17±0.31[b]
16:1ω7	1.21±0.07[b]	1.01±0.07	1.14±0.09	4.93±0.35	4.48±0.35	4.64±0.32	4.40±0.26	3.90±0.50	5.73±1.50	4.20±0.38	3.62±0.39	3.67±0.39
18:0	11.66±0.38	12.13±0.13	11.49±0.34	4.48±0.26	3.87±0.32	4.33±0.52	12.34±0.71	10.33±0.81	12.76±0.78[b]	1.33±0.11	1.58±0.25	1.40±0.09
18:1ω9	10.13±0.42	8.85±0.43	10.23±0.58	38.08±1.00	37.41±1.22	39.93±1.14	31.13±2.02	36.96±1.65	34.69±3.17	20.13±0.70	20.27±1.53	20.11±0.67
18:2ω6	23.18±0.90	24.10±0.73	21.82±1.23	15.76±1.10	18.97±1.03	15.19±0.80[c]	12.62±1.17	15.02±1.11	12.00±0.84[b]	49.33±1.56	49.12±1.51	49.82±1.56
20:3ω6	3.18±0.15	3.74±0.45	3.28±0.20	0.27±0.02	0.33±0.05	0.27±0.05	0.12±0.05	0.30±0.11	0.07±0.04[b]	0.79±0.06	0.72±0.13	0.70±0.09
20:4ω6	11.02±0.57	10.48±0.78	10.46±0.65	1.12±0.09	1.02±0.13	0.98±0.09	1.66±0.20	1.26±0.44	1.37±0.30	6.64±0.36	5.67±0.93	6.88±0.62
22:6ω3	2.46±0.13	2.20±0.25	3.32±0.43[b,d]	0.15±0.03	0.14±0.06	0.10±0.04	0.05±0.04	0.14±0.14	0.06±0.04	0.35±0.03	0.16±0.07	0.37±0.08

Values are mean ± SEM in % of total fatty acids.

[a]Number of samples, the subgroup of No MI with chronic anti-inflammatory treatment was excluded.

[b]Difference between MI and No MI or PGI extends to $p < 0.05$.

[c]Difference between MI and PGI extends to $p < 0.01$.

[d]Difference between No MI and PGI extends to $p < 0.05$.

TABLE 6

No MI vs MI Groups Compared with Respect to 10 Clinical Variables

Patient variable	No MI (n = 44)	MI (n = 14)	Test	p-Value
Use of prostaglandin inhibitors	19/44	0/14	Fisher's exact test	<0.002[a]
Average age[b]	69 ± 10	68 ± 12	Student's t-test	< 0.59
Sex	24M/20F	5M/9F	Fisher's exact test	<0.36
Arrhythmia	16/42	10/13	Fisher's exact test	<0.02[a]
Diabetes	6/41	0/12	Fisher's exact test	<0.32
Serum triglyceride average (mg/100 ml)[b]	167 ± 113	198 ± 121	Student's t-test	<0.40
Highest blood pressure (mm HG) (diastolic)[b]	88 ± 12	95 ± 15	Student's t-test	<0.07
Treated high blood pressure	16/44	4/14	Fisher's exact test	<0.75
Known coronary artery disease	25/44	7/14	Fisher's exact test	<0.76

[a]Statistically significant.

[b]Mean ± SD.

Patients who are having or have had MI seem to have higher NEFA than those who have symptoms of MI without permanent myocardial damage 12 hr after admission. Whether this difference in NEFA is a result of the MI or contributes to it cannot be answered by this study. However, it should be noted that elevation in blood NEFA has been related to platelet activation, increased infarction size and increased incidence of arrhythmias and death (1-4). Therapy aimed at decreasing NEFA in patients with MI has had some success (8).

Figure 1 shows a theoretical model that may explain how elevated NEFA could promote MI (11). Although serum nonesterified AA was not elevated in the MI patients, it is possible that the elevation in total NEFA may compete for albumin binding sites with AA and result in a local increase in unbound, nonesterified AA. This AA may be more active metabolically and could contribute to platelet aggregation. This may be the mechanism for NEFA elevation causing platelet aggregation described by Gerrard et al. (5). Increase in NEFA associated with fasting and with catecholamine release may explain the temporal association of increased platelet aggregability with the risk of MI and sudden cardiac death seen in the morning hours (12).

The precursors of polyunsaturated fatty acids (PUFA) in the NEFA fraction are the PL of tissue and plasma. The PUFA of the serum or plasma are indicators of the PUFA status of the individual (13), and profiles of these PUFA indicate abnormalities in PUFA metabolism that may occur. Figure 2 shows the PUFA profiles of serum PL, comparing the MI group with the Normal group and the No MI group with Normal group at the time of admission and again at 48 hr. At the time of admission, 20:3ω6 was significantly higher in both the MI and No MI groups. At 48 hr, 20:3ω6 remained high only in the MI group. AA was not significantly altered at the time of admission in either group but was elevated significantly in the MI group at 48 hr. The chain elongation product of AA, adrenic acid (22:4ω6), was normal at admission in both groups, but was very significantly elevated in the No MI group at 48 hr. The 22:5ω6 was elevated in the No MI group at admission and was significantly elevated in both groups at 48 hr. Of the ω3 PUFA, 22:6ω3 was elevated in the No MI group at admission and in both groups at 48 hr. The total ω3 acids in the MI group were 3.45% at admission, not significantly elevated above the Normal group value of 3.13%. They were significantly elevated ($p < 0.05$) at admission in the No MI group compared with the Normal group, 3.95% and 3.13%, respectively. The antithrombotic effects of ω3 acids may have contributed to the protection against cardiac damage in this group (14). The 20:3ω9, an indicator of essential fatty acid deficiency, was elevated significantly only in the No MI group at 48 hr. None of the changes in PUFA indicated deficiencies of ω6 or ω3 fatty acids. The elevation in the prostaglandin precursors, 20:3ω6 and 20:4ω6, in the MI group could have important physiological implications on the extent of myocardial damage present and clinical outcome for the patient.

The clinical observation was made in 1953 that aspirin appeared to protect against MI (15). Numerous studies since have been performed to assess the usefulness of prostaglandin inhibitors in the secondary prevention of MI.

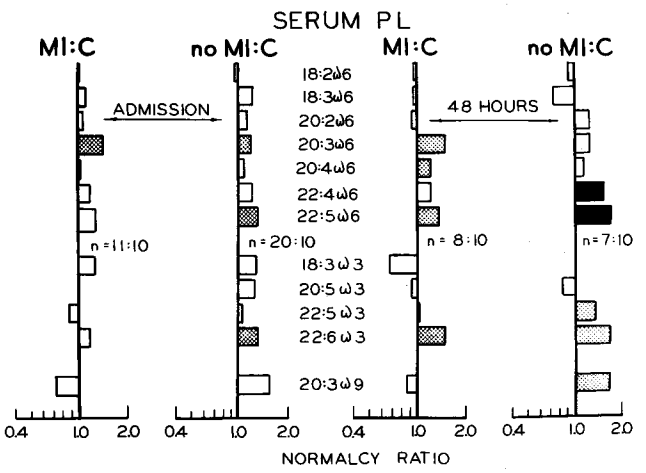

FIG. 2. Polyunsaturated fatty acid profiles for serum PL at admission and at 48 hr for MI and No MI groups. Normalcy ratio is experimental value divided by Normal value. Significance is indicated by shading. Open bars, non-significant; light crosshatch, $p < 0.05$; dark crosshatch, $p < 0.01$; black, $p < 0.001$.

Several studies have shown reduction in the rate of subsequent MI or coronary mortality from PGI (7,16-21). However, two large trials failed to show statistically significant benefit from prophylactic use of these drugs (6,22).

Our results are from an unselected population of patients presenting to an emergency room with chest pain or other clinical criteria requiring admission to rule out MI. There is an improved outcome for those taking a mixed variety of prostaglandin inhibitors (aspirin, ibuprofen, anproxen, prednisone, fenoprofen), most often for arthritis. The dose of these medications placed them in the high-dose prophylaxis category. Beneficial effects of prostaglandin inhibitors are believed to be due to reduced production of platelet TXA_2. Adverse effects may be the result of decreased levels of PGI_2 in the endothelial cell wall (23).

We considered an alternative explanation for our observations. Those taking prostaglandin inhibitors for arthritis might suffer from intermittent chest pain from their arthritis, for which they were admitted, to rule out MI. However, musculoskeletal chest pain is distinct from ischemic chest pain and always is considered in its differential diagnosis, so we think this explanation is unlikely. Coincidental effects from possible confounding risk factors for MI were evaluated as described above and did not account for this association.

In the setting of possible MI in this unselected group of emergency admissions, several clinical indices, some closely linked with coronary artery disease, were of no predictive value in identifying those patients with MI. This observation was true for age, sex and serum triglyceride and cholesterol levels. It was surprising that a prior history of treated diabetes mellitus, hypertension or coronary artery disease was of no value in predicting MI in our series. Our observations underscore the importance of prostaglandin-mediated pathophysiologic mechanisms in MI, but these effects are not mediated through alteration of NEFA levels, including AA, which showed no important difference between the four study groups.

In conclusion, NEFA are elevated in stressed patients admitted for possible MI. Those patients with MI seem to have marginally higher levels of NEFA than those with no MI. The MI, No MI and PGI groups all seem to have significantly different fatty acid patterns (with reduced percentage of AA) in NEFA compared with the Normal group NEFA-AA patterns. The fatty acid patterns in PL, NEFA, TG and CE were significantly different in the MI, No MI and PGI groups in a few fatty acids only. Significant changes in the PL occurred in both MI and No MI groups of patients compared with the Normal group, thereby implicating the effect of stress on fatty acid patterns.

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REFERENCES

1. Allison, S.P., Chamberlain, M.J., and Hinton, P. (1969) *Br. Med. J. 4,* 776–778.
2. Oliver, M.F., Kurien, V.A., and Greenwood, T.W. (1968) *Lancet 1,* 710–714.
3. Ylikahri, R.H. (1977) *Ann. Clin. Res. 9,* 102–111.
4. Tansey, M.J., and Opie, L.H. (1983) *Lancet 2,* 419–422.
5. Gerrard, J.M., White, J.G., and Krivit, W. (1974) *Blood 44,* 918.
6. Elwood, P.C., Cochrane, A.L., Burr, M.L., Sweetman, P.M., Williams, G., Hughes, S.J., and Renton, R. (1974) *Br. Med. J. 1,* 436–440.
7. The Coronary Drug Project Research Group (1976) *J. Chronic Dis. 29,* 625–642.
8. Rackley, C.E., Russel, R.O. Jr., and Rogers, W.J. (1979) *Postgrad. Med. 65,* 93–99.
9. Mueller, H.S., and Ayres, S.M. (1980) *J. Clin. Invest. 65,* 338–346.
10. McDaniel, H.G., Papapietro, S.E., Rogers, W.J., Mantle, J.A., Smith, L.R., Russell, R.O. Jr., and Rockley, C.E. (1981) *Am. Heart J. 102,* 10–15.
11. Ogburn, P.L. Jr., and Brenner, W.E. (1981) in *Current Concepts,* p. 39, Upjohn Company, Kalamazoo, MI.
12. Tofler, G.H., Brezinski, D., Schafer, A.I., Czeisler, C.A., Rutherford, J.D., Willich, S.N., Gleason, R.E., Williams, G.H., and Muller, J.E. (1987) *N. Eng. J. Med. 316,* 1514–1518.
13. Holman, R.T. (1986) *Am. J. Coll. Nutr. 5,* 183–211.
14. Connor, W.E., Harris, W.S., and Goodnight, S.H. Jr. (1983) in *Dietary Fats and Health* (Perkins, E.G., and Visch, W.J., eds.) pp. 518–529, American Oil Chemists' Society, Champaign, IL.
15. Craven, L. (1953) *Miss. Valley Med. J. 75,* 38–44.
16. Breddin, K., Loew, D., Lechner, K., Uberla, K., and Walter, E. (1979) *Thromb. Haemost. 41,* 225–236.
17. Elwood, P.C., and Sweetman, P.M. (1979) *Lancet 2,* 1313–1315.
18. The Persantine-Aspirin Reinfrarction Study Group (1980) *Circulation 62,* 449–461.
19. The Anturane Reinfarction Trial Research Group (1978) *N. Eng. J. Med. 298,* 289–295.
20. The Anturane Reinfarction Trial Research Group (1980) *N. Eng. J. Med. 302,* 250–256.
21. The Anturane Reinfarction Italian Study Group (1982) *Lancet 1,* 237–242.
22. Aspirin Myocardial Infarction Study Research Group (1980) *J. Am. Med. Assoc. 243,* 661–669.
23. Beamish, R.E., Das, P.K., Karmazyn, M., and Dhalla, N.S. (1985) *Can. J. Cardiol. 1,* 66–74.

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Microsomal Desaturation-Elongation of Linoleic Acid Following Parenteral Feeding with Lipid Emulsions in the Rat

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The effect of total parenteral nutrition (TPN) with lipid emulsion to supply either 27.5% or 2% total calories on in vitro desaturation-elongation of 18:2(n-6) by liver microsomes was studied in the rat. The emulsion lipid contained ca. 50% 18:2(n-6) plus 8% 18:3(n-3) or 77% 18:2(n-6) plus 0.5% 18:3(n-3). The reaction rate was influenced by the in vitro substrate concentration and inhibited above 50 μmol 18:2(n-6)/mg microsomal protein. At maximum rates of desaturation-elongation, the formation of triene and tetraene (n-6) fatty acids was reduced in rats given either of the two emulsions. The rate of (n-6) pentaene formation was increased in rats given the emulsion with low 18:3(n-3) but not in rats given the emulsion with 8% 18:3(n-3). Analyses of the microsomal lipid indicated increased free cholesterol in all rats that received TPN. Esterified cholesterol was increased only in rats given 27.5% TPN calories as lipid. Microsomal total phospholipids and phospholipid class distributions were not altered by TPN. The data are consistent with reports of reduced levels of long-chain desaturation-elongation metabolites of 18:2(n-6) in tissue phospholipids following infusion of parenteral lipid. The data suggest that the mechanism may include alterations in other metabolic pathways such as cholesterol, in addition to desaturase enzyme activities, and may be influenced by both the quantity and fatty acid composition of the lipid infused. ***Lipids 23,*** **546–550 (1988).**

The composition of diet fat is known to influence membrane structural lipid fatty acid composition and metabolic function (1), as well as lipoprotein (2) and eicosanoid (3) metabolism. Desaturation-elongation pathways for conversion of dietary essential fatty acids, linoleic acid (18:2[n-6]) and linolenic acid (18:3[n-3]), to their more highly unsaturated homologues, which are incorporated into tissue phospholipid and serve as eicosanoid precursors, have been studied extensively (4,5). Competition among C18 (n-6) and (n-3) fatty acids for Δ6 desaturation is well known, as is the inhibition of desaturation caused by various substrates and reaction products (4–9).

Artificial fat emulsions are used widely to supply a major portion of the calories infused during IV nutritional support (total parenteral nutrition, TPN). Commonly used formulations are based on soy oil or safflower oil emulsified with egg phospholipids and contain 45% or more 18:2(n-6) and up to 8% 18:3(n-3) in total fatty acids. Their infusion provides a substantially higher intake of 18:2(n-6) and markedly different fatty acid balance to that encountered in usual human diets. Recent reports have described decreased C20 and C22 (n-6) and (n-3) fatty acids in tissue phospholipids of infants and rats given TPN with lipid emulsion (10,11). Decreased 20:4(n-6) availability also has been suggested to explain the reduced levels of some eicosanoid metabolites found in infants given parenteral lipid emulsions (12). The effects of these emulsions on essential fatty acid metabolism has not been studied directly. Thus, the purpose of the study reported here was to investigate the effect of TPN with different 18:2(n-6) contents on hepatic microsomal desaturation-elongation of 18:2(n-6) in the rat.

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Abbreviations: GLC, gas liquid chromatography; TLC, thin layer chromatography; TPN, total parenteral nutrition.

MATERIALS AND METHODS

Materials. [1-^{14}C]Linoleic acid (40–60 mCi/mmol) was from New England Nuclear (Montreal, Quebec). Cofactors used in the enzymatic assay were from Sigma Chemical Co. (St. Louis, MO). Fatty acid methyl esters used as carriers and for gas liquid chromatography (GLC) standards were from Supelco Inc. (Bellefonte, PA) and from NuChek-Prep Inc. (Elysian, MN). The purity of standards and the radiolabeled substrate was confirmed by GLC and argentation thin layer chromatography (TLC), respectively. TPN solutions were prepared as described (13,14) using 50% dextrose, parenteral amino acids (Travenol, 10%, Baxter Labs., Malton, Ontario), vitamins (MVI-12, USV Canada Inc., Mississauga, Ontario) and minerals (Addamel, Pharmacia Canada Inc., Dorval, Quebec). Liposyn (10%) was from Abbott Labs (Montreal, Quebec) and Intralipid (10% Nutralipid) was from Pharmacia. The fatty acid composition of the emulsions and the chow diet has been reported (10); Liposyn and Intralipid contained ca. 77% and 0.5%, and 50% and 8% 18:2(n-6) and 18:3(n-3), respectively. The rat chow contained ca. 4% fat with 34% 18:2(n-6) and 3% 18:3(n-3) (10).

Animals and TPN. Rats (male Wistar, 175–225 g) were surgically prepared for TPN (13) and infused for seven days. The infusate contained ca. 27.5% calories as lipid from Liposyn or Intralipid, or 2% calories from Intralipid with 25.5% calories from additional dextrose, six rats each. The remaining 72.5% calories were supplied by the amino acid-dextrose solution (13,14). Six more rats were sham-operated and allowed free access to Purina rat chow (control). The diets provided ca. 1.4, 1.0, 13.7, and 21.2% calories as 18:2(n-6) and ca. 0.1, 0.2, 2.3 and 0.1% calories as 18:3(n-3) in the control, 2% Intralipid, 27.5% Intralipid and 27.5% Liposyn groups, respectively. The outcome of TPN was similar to previous studies (10,13,15). All rats completed the course of TPN with normal results in plasma tests of liver function and with maintenance of body weight over the infusion period.

Preparation of liver microsomes. The rats were anaesthetized (0900–1000 hr, sodium pentothal, 0.4 mg/kg); the livers excised and immediately rinsed in saline, then in a 0.15 M KCl, 5 mM $MgCl_2$, 0.25 M sucrose, 1.5 mM glutathione, 50 mM potassium phosphate buffer, pH 7.4. Tissue portions were homogenized in 2 vol. buffer, centrifuged, 10,000 g × 20 min and the microsomes recovered from the supernatant by centrifugation, 100,000 g × one hr, without washing (16). All isolation procedures were conducted at 0–4 C. Microsomal protein recovery was

determined by the method of Lowry et al. (17) with bovine serum albumin as the standard.

Desaturation-elongation of 18:2(n-6). Incubations were performed in 1 ml of 0.15 M KCl − 0.05 M KPO_4 buffer (pH 7.0) containing 1.5 mM glutathione, 5 mM ATP, 1.25 mM NADH, 1.25 mM NADPH, 0.3 mM CoA, 0.3 mM malonyl CoA essentially as described by Purvis et al. (18) and using 0.5–3.0 mg microsomal protein. The reactions were started by addition of substrate, 5–200 μmol 18:2(n-6) with 0.1 μCi [^{14}C]18:2(n-6), and continued 20 min at 37 C. They were stopped and saponified with 1.0 ml 20% (w/v) KOH, acidified with 2 N HCl and the fatty acids extracted (19), methylated with methanolic HCl and then dried under N_2. The resulting methyl esters were dissolved in petroleum ether (bp 30–60 C) with a mixture of unlabeled methyl ester carriers (100 μg each) and then separated according to their unsaturation by argentation TLC on Silica Gel G impregnated with 10% $AgNO_3$ (20). The plates were developed three times at −20 C in toluene acetone (95:5, v/v) and the bands containing saturated, mono-, di-, tri-, tetra- and pentaenoic fatty acids visualized under UV light after spraying with 2′7′-dichlorofluorescein (0.1%, w/v). [^{14}C] in each band was quantitated by liquid scintillation spectrometry with quench correction and conversion to dpm. Recovery of radioactivity from the TLC plates was >95%.

Microsomal lipid composition. Lipids were extracted (19) from a known amount of microsomal protein and aliquots taken for determination of total lipid phosphorous (21), total phospholipid fatty acid composition by GLC (10) and free and esterified cholesterol content (22), following their separation on Silica Gel G utilizing petroleum ether/diethyl ether/acetone (85:15:3, v/v/v). Phospholipid classes were quantitated (21) following separation according to Skipski et al. (23).

Data handling. Enzyme activities were calculated as the nmol fatty acid substrate converted per mg protein per min. Because this may underestimate absolute conversion rates to the extent that the labeled substrate was diluted by the microsomal substrate pool, the conversion of 18:2(n-6) to tri-, tetra- and pentaene products also was calculated as percent conversion. Microsomal fatty acid peak areas, weight percentage and μg values from GLC analyses were determined by a chromatography data system (10). Statistical analysis for treatment differences utilized analyses of variance and Duncan's multiple-range test.

RESULTS AND DISCUSSION

Desaturation-elongation of 18:2(n-6) by normal rat liver microsomes. The conversion of C18 fatty acids to higher homologues and their incorporation into tissue lipid involves several complex reactions that potentially may be altered by reaction substrates and/or products. In vitro kinetic analyses have indicated the regulatory importance of desaturation by demonstration that the rate of fatty acid activation to the CoA thioester, acyl-CoA elongation, and transfer and incorporation into lipid products all exceed the rate of the respective desaturation (4,5). Thus, experimental (10) and clinical (11,12) evidence of reduced tissue levels of highly unsaturated metabolites of 18:2(n-6) and 18:3(n-3) following parenteral lipid infusion may suggest inhibition of microsomal desaturation. These studies used a coupled desaturation-elongation assay rather than the more usual study of individual desaturases with single fatty acyl substrates and no elongation cofactors or malonyl-CoA, as more analogous to in vivo metabolism in which n-6 fatty acid substrates for Δ5- and Δ4-desaturation are largely derived from Δ6-desaturation of (dietary) 18:2(n-6).

The total desaturation of 18:2(n-6), derived as the sum of all triene, tetraene and pentaene methyl ester products, together with the rate of triene, tetraene and pentaene formed and remaining in the respective fraction is shown in Figure 1. The Δ6 desaturase represents the first enzyme in the sequence, thus the total activity of this enzyme is reflected by the sum of all desaturation products. The reaction rates were linear with 0.5–3.0 mgm microsomal protein at all substrate concentrations (data not shown) but were markedly reduced at substrate concentrations above 50 μmol. The finding of maximum desaturation-elongation rate at 50 μmol 18:2(n-6)mg/protein agrees with previous specific assays of rat liver Δ6 desaturase (24,25). The reaction rates achieved also are similar to the Δ6-desaturase activities reported by others using similar substrate levels (16,24,26,27). The lower rate of desaturation-elongation at 200 μmol substrate (control group, 0.28 ± 0.07 vs 0.32 ± 0.02 nmol/min/mg protein with 200 vs 50 μmol 18:2(n-6) substrate, respectively) is similar to Δ6-desaturase activity reported by Alam et al. (28) with this substrate concentration.

The distribution of [^{14}C] in fatty acid methyl esters following desaturation-elongation of 18:2(n-6) is shown in

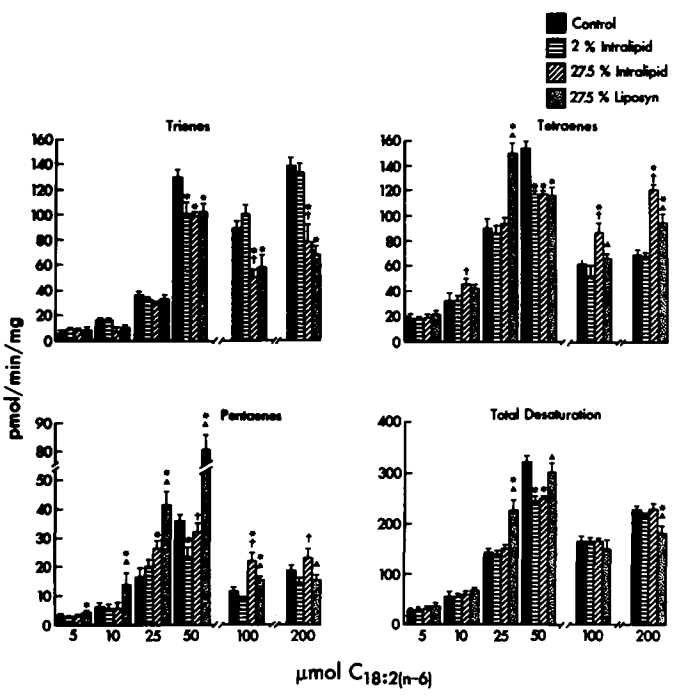

FIG. 1. Desaturation of 18:2(n-6) by rat liver microsomes following seven days TPN with 2% calories from Intralipid or 27.5% calories from Intralipid or Liposyn. The reactions were conducted with 5–200 μmol 18:2(n-6) substrate and 1 mg microsomal protein and performed in triplicate. The results represent mean ± SE for six rats, with significant differences of $p \leq 0.05$ indicated as *, TPN vs control; †, 27.5% Intralipid vs 2% Intralipid; ▲, 27.5% Liposyn vs 27.5% Intralipid.

Figure 2 and reflects the relative activities of the three desaturases studied. The relative activities of Δ6-, Δ5- and Δ4-desaturase with (n-6) series fatty acids has been calculated as 1.0:1.0:0.02, respectively (29), suggesting that the slowest enzyme in the sequence is Δ4-desaturase. Rat liver microsomes have been shown to have very low Δ4-desaturase activity with (n-6) fatty acyl substrates (30). The recovery of over 85% [^{14}C] as triene and tetraene fatty acid methyl esters in these studies (Fig. 2) is consistent with a slow conversion of 22:4(n-6) to 22:5(n-6).

At in vitro substrate concentrations up to apparent saturation (50 μmol 18:2[n-6]), the recovery of [^{14}C] was higher in tetraene than triene methyl esters (Fig. 2). This pattern suggests that the activity of Δ5-desaturase was similar or higher than that of Δ6-desaturase. Previous in vitro work utilizing low 18:2(n-6) and 20:3(n-6) substrate concentrations also have found higher rates of Δ5- than Δ6-desaturation (27,31). In contrast, at substrate concentrations above the apparent reaction optimum the relative distribution of [^{14}C] showed accumulation in triene rather than tetraene methyl esters (Fig. 2), possibly suggesting inhibition of desaturation at the Δ5-desaturation step.

Influence of TPN 18:2(n-6) desaturation-elongation. The maximum rate of 18:2(n-6) desaturation-elongation attained, and the formation of triene and tetraene fatty acids, was decreased in rats that had received 2 or 27.5% TPN calories from Intralipid (Fig. 1). In contrast, the provision of 27.5% TPN calories as Liposyn had no effect on total rates of desaturation-elongation, although the formation of triene and tetraene fatty acids was reduced similarly. The higher rate of total desaturation in the groups given Liposyn compared with the groups given Intralipid was due to a marked increase in the rate of pentaene fatty acid synthesis. This apparent induction of Δ4-desaturation was observed in the assays with microsomes prepared from rats given Liposyn at all nonsaturating 18:2(n-6) substrate levels (Fig. 1). Whether the difference between the emulsions is related to their markedly different 18:2(n-6)/18:3(n-3) ratios (ca. 5.6 and 154 in Intralipid and Liposyn, respectively) is unknown. Diets very low in 18:3(n-3) that produce low tissue levels of 22:5(n-3) and 22:6(n-3) have been associated with increased levels of 22:5(n-6) (32). Definitive evidence of increased Δ4-desaturation of (n-6) fatty acids with such diets is not available. The total calories received as 18:3(n-3) was similar among the control, 2% Intralipid and 27.5% Liposyn groups. Thus, it appears likely that the fatty acid balance rather than absolute intake of 18:3(n-3) may be important.

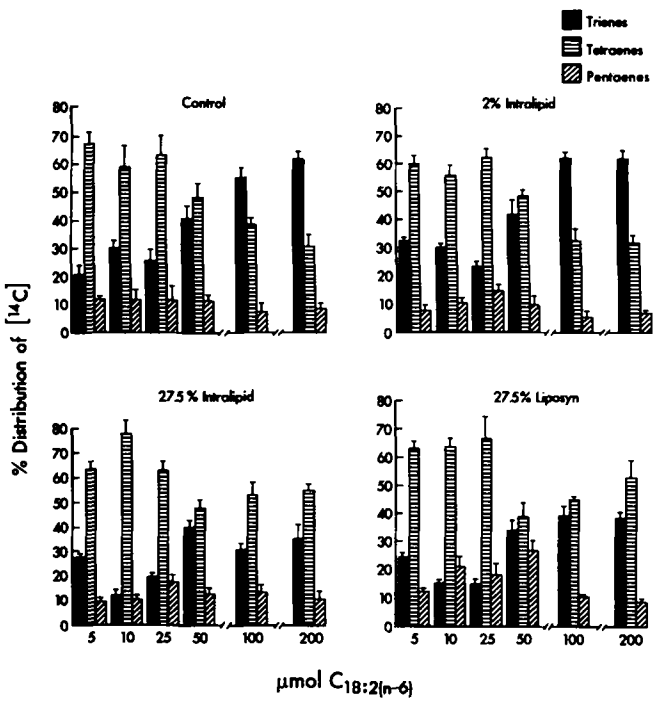

FIG. 2. Recovery of [^{14}C] 18:2(n-6) in triene, tetraene or pentaene fatty acid products expressed as % of total desaturation by rat liver microsomes following seven days TPN with 2% calories from Intralipid or 27.5% calories from Intralipid or Liposyn. The reactions were conducted with 5–200 μmol 18:2(n-6) substrate and 1 mg microsomal protein and performed in triplicate. Results represent means ± SE for six rats.

The distribution of [^{14}C] among triene, tetraene and pentaene methyl ester products in the three TPN groups was similar to that in the control group up to 50 μmol substrate (Fig. 2). In contrast with the control and 2% Intralipid groups this pattern, which suggests higher Δ5-, than Δ6- or Δ4-desaturation, persisted at the higher substrate concentrations in both groups given 27.5% TPN calories as lipid. The difference between the use of 2% and 27.5% calories from parenteral lipid implies the effect was due to the quantity of parenteral lipid provided rather than TPN itself.

Influence of TPN on rat hepatic microsomal lipids. Consideration of the microsomal lipid composition is of interest because of reports that microsomal membrane fluidity may regulate desaturase enzyme activities (33–35), and because phospholipid fatty acids in situ, in addition to fatty acyl-CoA, may serve as substrates for these enzymes (31). Analysis of the microsomal lipid showed increased free cholesterol, and an increased molar cholesterol/phospholipid ratio in all rats that had received TPN, irrespective of its composition or quantity (Table 1). The accumulation of free cholesterol was greater with 27.5% than 2% Intralipid or 27.5% Liposyn. Esterified cholesterol levels were increased and decreased in groups that had received 27.5% and 2% calories as lipid, respectively (Table 1). Phospholipid class distributions were not altered by TPN (control values, mean ± SE as percent total phospholipid, phosphatidylcholine 57.8 ± 2.0, phosphatidylethanolamine 23.6 ± 0.4, phosphatidylserine + phosphatidylinositol 13.8 ± 0.8, sphingomyelin 4.6 ± 0.3).

The elevation of microsomal membrane cholesterol following TPN may involve several mechanisms including altered hepatic lipid metabolism (36) or bile acid excretion (14). This membrane is the site of cholesterol 7α-hydroxylase, which utilizes membrane-free cholesterol for synthesis of bile acid. Whether reduced hepatic bile acid secretion following TPN could impair maintenance of microsomal cholesterol homeostasis is unknown. However, previous studies reported greater reduction of bile acid secretion in rats infused with Intralipid than with Liposyn (14) and thus are consistent with the data in this study, indicating that high levels of Intralipid gave the greatest accumulation of microsomal-free cholesterol (Table 1). Similarly, cholestasis of other etiologies has been shown to result in free cholesterol accumulation in hepatic membranes in this species (37).

The description in previous studies of reduced total hepatic and plasma phospholipid C20 and C22 (n-6) and (n-3) fatty acids in rats (10) and human infants (11) who had received parenteral lipid were not clearly reflected in the microsomal phospholipid fatty acids in this study (Table 2). Rats that had received 2% calories as Intralipid had increased 16:0 and 20:3(n-6) and decreased 18:2(n-6) and 22:3(n-3) levels. Infusion of 27.5% calories from Intralipid or Liposyn increased the microsomal 18:0 and 18:2(n-6) content. Intralipid, but not Liposyn, at this level also reduced the 20:4(n-6) and 22:5(n-3) levels when compared with the control values. The lipid composition of biomembranes is membrane- and tissue-specific and depends on desaturase activities as well as more complex mechanisms, including preferential acylation and deacylation/reacylation reactions (38). Thus, changes in a specific membrane may not reflect desaturase activities or those of other membrane sites. Conceivably, specificity of acylation and remodeling may explain differences between the effects of TPN on microsomal phospholipids (Table 1) and total liver phospholipids (10). The finding that the rate of triene and tetraene fatty acid formation from 18:2(n-6) was reduced under maximum in vitro reaction rate conditions after infusion of 27.5% calories as either Intralipid

TABLE 1

Lipid Composition of Rat Hepatic Microsomes Following Seven Days of TPN

		TPN		
	Control	2% Intralipid	27.5% Intralipid	27.5% Liposyn
Phospholipid (nmol/mg protein)	495 ± 20	433 ± 38	421 ± 19	455 ± 37
Free cholesterol (nmol!mg protein)	45.8 ± 1.0	74.4 ± 3.4[b]	95.6 ± 3.8[b,d]	73.5 ± 4.8[b,e]
Esterified cholesterol (nmol!mg protein)	3.7 ± 0.4	1.5 ± 0.2[b]	6.4 ± 0.8[a,b]	6.7 ± 0.6[b]
Cholesterol/phospholipid (nmol/mol)	0.10 ± 0.01	0.18 ± 0.02[b]	0.24 ± 0.01[b,c]	0.18 ± 0.03[b]

Values given represent means ± SE, n = 6 for all groups.

[a,b]Values for TPN groups are significantly different ($p < 0.05$, 0.01, respectively) from the control.

[c,d]Values for 27.5% Intralipid are significantly different ($p < 0.05$, 0.01, respectively) from the 2% Intralipid group.

[e]Value for 27.5% Liposyn significantly different, $p < 0.01$, from the 27.5% Intralipid group.

TABLE 2

Major Fatty Acids of Rat Hepatic Microsomal Phospholipids Following Seven Days of TPN

Fatty acids (% w/w)		TPN		
	Control	2% Intralipid	27.5% Intralipid	27.5% Liposyn
14:0	0.8 ± 0.2	0.2 ± 0.0[a]	0.2 ± 0.1	0.2 ± 0.1
16:0	20.0 ± 0.6	25.8 ± 0.4[b]	19.1 ± 0.8[f]	19.4 ± 1.0
16:1	1.7 ± 0.3	2.8 ± 1.1	1.7 ± 0.8	0.7 ± 0.3
18:0	25.9 ± 0.6	25.4 ± 1.4	29.4 ± 1.4[a]	31.0 ± 1.5[b]
18:1	11.1 ± 1.4	9.7 ± 1.3	10.0 ± 2.1	7.4 ± 1.1
18:2	13.7 ± 0.7	11.9 ± 1.8	16.6 ± 0.8[a,c]	15.6 ± 0.4[a]
18:3(n-3)	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
20:3(n-6)	0.4 ± 0.0	0.9 ± 0.1[b]	0.4 ± 0.1[d]	0.4 ± 0.1
20:4(n-6)	16.6 ± 1.3	14.3 ± 1.0	13.5 ± 0.3[a]	17.1 ± 1.0[f]
20:5(n-3)	0.3 ± 0.0	0.8 ± 0.3	0.2 ± 0.0	0.1 ± 0.0[a]
22:3(n-3)	0.2 ± 0.0	0.0 ± 0.0[a]	0.2 ± 0.2	0.1 ± 0.1
22:4(n-6)	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.2	0.3 ± 0.0[a]
22:5(n-6)	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.1[e]
22:5(n-3)	0.6 ± 0.1	0.9 ± 0.3	0.4 ± 0.1	0.4 ± 0.0[a]
22:6(n-3)	4.7 ± 0.4	4.6 ± 0.1	4.0 ± 0.8	3.8 ± 0.4

Data given represent means ± SE, n = 6 for all groups.

[a,b]Values for TPN group are significantly different ($p < 0.05$, 0.01, respectively) from the control.

[c,d]Values for 27.5% Intralipid are significantly different ($p < 0.05$, 0.01, respectively) from the 2% Intralipid group.

[e,f]Values for 27.5% Liposyn are significantly different ($p < 0.05$, 0.01, respectively) from the 27.5% Intralipid group.

or Liposyn, however, is consistent with the previous demonstration of reduced hepatic phosphatidylcholine 20:3(n-6) and 20:4(n-6) in similarly treated rats (10). Also of relevance are reports that diet-induced elevation of plasma cholesterol is associated with reduced plasma and liver 20:4(n-6)/18:2(n-6) and 22:6(n-3)/20:5(n-3) (39). The infusion of parenteral lipid is known to lead to accumulation of plasma-free cholesterol (40,41), which appears to extend to liver cell membranes (Table 2). Thus, it is possible that alterations in cholesterol metabolism caused by parenteral lipid emulsions are related to changes in plasma and tissue in (n-6) and/or (n-3) fatty acid levels.

TPN with lipid emulsions continues to be invaluable for the provision of calories to critically ill patients and those with compromised gastrointestinal function. Clinical (11) and animal (10) studies concur in their demonstration of reduced C20 and C22 (n-6) and (n-3) fatty acids in tissue phospholipids following infusion of these products. Although a definitive reason is unknown, it appears likely that it may include altered desaturase enzyme activities and changes in other aspects of hepatic lipid and cholesterol metabolism. The evidence suggests that the mechanism is influenced by both the quantity and fatty acid composition of the parenteral lipid infused.

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REFERENCES

1. Stubbs, C.D., and Smith, A.D. (1987) *Biochim. Biophys. Acta 779*, 89-137.
2. Mattson, F.H., and Grundy, S.M. (1985) *J. Lipid Res. 26*, 194-202.
3. Uotila, P., and Vapaatalo, H. (1984) *Ann. Clin. Res. 16*, 226-233.
4. Brenner, R.R. (1981) *Prog. Lipid Res. 20*, 41-47.
5. Jeffcoat, R., and James, A.T. (1984) in *Fatty Acid Metabolism and Its Regulation* (Numa, S., ed.) pp. 85-112, Elsevier Publishing, New York.
6. Brenner, R.R. (1974) *Mol. Cell. Biol. 3*, 41-52.
7. Brenner, R.R., Gorda, H., de Gomez Dumm, I.N.T., and Pezzano, H. (1981) *Prog. Lipid. Res. 20*, 315-321.
8. Marcel, Y.L., Christiansen, K., and Holman, R.T. (1968) *Biochim. Biophys. Acta 164*, 25-34.
9. Nervi, A.M., Brenner, R.R., and Peluffo, R.O. (1968) *Biochim. Biophys. Acta 152*, 539-551.
10. Innis, S.M. (1986) *Lipids 21*, 132-138.
11. Martinez, M., and Ballabriga, A. (1987) *Lipids 22*, 133-138.
12. Friedman, Z., and Frohlich, J. (1979) *Pediatr. Res. 13*, 932-936.
13. Innis, S.M., and Boyd, M.C. (1983) *Am. J. Clin. Nutr. 38*, 95-100.
14. Innis, S.M. (1985) *Am. J. Clin. Nutr. 41*, 1283-1288.
15. Innis, S.M. (1986) *J. Pediatr. Gastroenterol. Nutr. 5*, 793-798.
16. Leikin, A.I., and Brenner, R.R. (1986) *Biochim. Biophys. Acta 876*, 300-308.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265-275.
18. Purvis, J.M., Clandinin, M.T., and Hacker, R.R. (1983) *Comp. Biochem. Physiol. 75B*, 199-204.
19. Folch, J., Lees, M., and Stanley, G.H.S. (1957) *J. Biol. Chem. 226*, 497-509.
20. Svetashev, V.I., and Zhukova, N.V. (1985) *J. Chromatogr. 330*, 396-399.
21. Ames, B.N. (1956) *Anal. Chem. 28*, 115-118.
22. Zak, B., Dickenman, R.C., White, E.G., Burnett, H., and Cherney, P.J. (1954) *J. Biol. Chem. 24*, 1307-1310.
23. Skipski, V.P., Peterson, R.F., and Barclay, M. (1964) *Biochem. J. 90*, 374-377.
24. Mahfouz, M.M., Johnson, S., and Holman, R.T. (1980) *Lipids 15*, 100-107.
25. Ullman, D., and Sprecher, H. (1971) *Biochim. Biophys. Acta 248*, 61-70.
26. Jeffcoat, J., and James, A.T. (1977) *Lipids 12*, 469-474.
27. Mahfouz, M.M., Smith, T.L., and Kummerow, F.A. (1984) *Lipids 19*, 214-222.
28. Alam, S.Q., Alam, B.S., and Chen, T.-W. (1984) *Biochim. Biophys. Acta 792*, 110-117.
29. Sprecher, H. (1977) *Adv. Exp. Med. Biol. 83*, 35-50.
30. Hagve, T.-A., Christophersen, B.O., and Dannerig, B.H. (1986) *Lipids 21*, 202-205.
31. Pugh, E.L., and Kates, M. (1984) *Lipids 19*, 48-55.
32. Kramer, J.K.G., Farnsworth, E.R., Thompson, B.K., and Corner, A.H. (1981) *Prog. Lipid Res. 20*, 491-499.
33. Garda, H.A., and Brenner, R.R. (1984) *Biochim. Biophys. Acta 769*, 160-170.
34. Garda, H.A., and Brenner, R.R. (1985) *Biochim. Biophys. Acta 819*, 45-54.
35. Kurata, N., and Privett, O.S. (1980) *Lipids 15*, 512-518.
36. Hall, R.I., Grant, J.P., Ross, L.H., Coleman, R.A., Bozovic, M.G., and Quarfordt, S.H. (1984) *J. Clin. Invest. 74*, 1658-1668.
37. Phillips, M.J., Poucell, S. and Oda, M. (1986) *Lab. Invest. 54*, 593-608.
38. Huang, Y.S., Nassar, B.A., and Horrobin, D.F. (1986) *Biochim. Biophys. Acta 879*, 22-27.
39. Breckenridge, W.C., Kakis, G., and Kuksis, A. (1979) *Can. J. Biochem. 57*, 72-82.
40. Griffin, E., Breckenridge, W.C., Kuksis, A., Bryan, M.H., and Angel, A. (1979) *J. Clin. Invest. 64*, 1703-1712.
41. Bell, R.M., and Coleman, R.A. (1980) *Annu. Rev. Biochem. 49*, 459-487.

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Isolation and Chemical Composition of Surface-active Material from Human Lung Lavage

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Surface-active material (SF) was isolated from human lung lavage fluid collected at autopsy employing differential and sucrose density gradient centrifugation. The isolated material showed well-defined electron microscopic structure, consisting of clearly preserved, closely packed vesicles with limiting membranes and inclusion bodies. It showed a very high degree of alkaline phosphatase specific activity and was devoid of other subcellular contaminants. The isolated material also showed a high phospholipid/protein ratio and increasing surface activity when monitored at different stages of purification. It contained 68.5% phosphatidylcholine, 11.5% phosphatidylglycerol and relatively smaller amounts of phosphatidylethanolamine and other individual phospholipid (PL) classes. In addition, cholesterol, unesterified fatty acids, triacylglycerols and other neutral lipids were found. Saturated fatty acids, particularly palmitic acid (16:0), predominated in the major PL fractions. However, various fatty acids of which oleic acid (18:1) constituted a large proportion also are present. Chemical analysis of the material showed that besides lipids and proteins, nucleic acids, sialic acid, hexose, amino sugars, nitrogen and phosphorus were present. The delipidated material showed the presence of three to four proteins as characterized by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis, and gel permeation chromatography on Sephadex G-200 resolved two well-separated peaks. The first fraction contained serum-associated 68 kDa protein, while the second fraction had two apoproteins with molecular weights of 34 kDa and 10 kDa. These two proteins were associated with the SF and they, as well as the whole surface-active material, strongly reacted with the antibody directed against the whole SF in a double-diffusion immunoprecipitation assay. The first Sephadex fraction containing a 68 kDa protein did not produce any precipitation line when reacted against antisera.
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The lung biosynthesizes a surface-active material called pulmonary surfactant (SF), which lines the surface of the alveoli and prevents collapse or atelactasis due to the pressure differences at air-water interfaces during deflation (1). Chemically, SF is a lipid-protein complex (2) that is required to maintain the normal mechanical properties of the lung and is secreted by type-II epithelial cells in the lung (3). Clinically, insufficient SF production may cause respiratory distress syndrome (RDS) and formation of hyaline membranes (4). Therefore, it is the main cause of mortality and morbidity in prematurely delivered neonates. Not knowing the chemical composition of this material, efforts to study its cellular synthesis, secretion and possible therapeutic approach in RDS have been seriously impeded.

In this paper, we describe a simple method for isolation and purification of SF from human endobronchial lavage fluid collected at autopsy, using a combination of differential and density gradient centrifugation. We also report the results of chemical, immunological and physical analyses of the isolated product. Partial characterization of selected enzymes and the SF-associated apoproteins also are presented. The development of an easy, rapid procedure for large-scale isolation of this unique lipoprotein complex is extremely important for the biophysical and biochemical understanding of its biosynthesis, assembly, secretion and adsorption to the alveolar interfaces. It also is important for developing immunoassay methods for quantitation and prenatal assessment of surfactant and, therefore, lung maturation. Prediction of SF deficiency in the prematurely born neonates will allow timely therapeutic infusion of synthetic surfactants.

MATERIAL AND METHODS

Isolation of pulmonary SF. Adult human lungs of both sexes at autopsy were obtained within two to four hr after death from non-pulmonary causes from the hospital attached to this institute. No patients had alveolar proteinosis, and samples visibly contaminated with blood were discarded. The excised lungs were separately lavaged by infusing chilled buffer A (0.005 M Tris – HCl, pH 7.35, containing 0.15 M NaCl). They were gently massaged, and the buffer was withdrawn by pouring back into a beaker, usually with 60–70% recovery. Thereafter, all the operations were performed strictly at 4 C. Cells and membraneous debris were removed from this crude lavage fluid by centrifugation at 160 × g for 10 min, and the materials suspended in the supernatant were pelleted by further centrifugation at 40,000 × g for two hr in a Sorvall SS-34 rotor. The sediment, a brownish precipitate, was suspended in and osmotically equilibrated with buffer A for two hr. About 30 ml of this suspension were placed in 50 ml centrifuge tubes and underlayered with 10 ml of 0.75 M sucrose, prepared in buffer A. After centrifugation at 18,000 × g for 10 min, the white interface was collected and, after dilution with four vol of buffer A, it was pelleted at 18,000 × g for 30 min. The resultant pellet was suspended in buffer A, and about 10 ml of it was underlayered with 12 ml each of a stepwise gradient consisting of 0.65 and 0.41 M sucrose, prepared in buffer A. The gradients were centrifuged at 75,000 × g for one hr in a SW-28 swinging bucket rotor in a Beckman ultracentrifuge maintained at 4 C. Purified SF appeared as a major band at the buffer A/0.41 M sucrose interface

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Abbreviations: AKP, alkaline phosphatase; DPPC, dipalmitoyl phosphatidylcholine; EDTA, ethylenediamine tetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipid; SDS, sodium dodecyl sulfate; SF, pulmonary surfactant; EM, electron microscopic characterization; GLC, gas liquid chromatography; MSE, probe-type sonicator; TLC, thin layer chromatography; RDS, respiratory distress syndrome.

(fraction I) and a minor very fine band at 0.41 M/0.61 M sucrose interface (fraction II). Both fractions were collected, diluted with four vol of buffer A and pelleted as above. By electron microscopic characterization (EM), glycerophospholipid analysis and surface-active properties (2), fraction I was the major surfactant material and was further characterized.

Surface tension determination. Surface activity of the SF suspensions was measured with a pulsating bubble surfactometer (Surfactometer International, Toronto, Canada), as described by Enhorning (5). A bubble with ambient air was created by withdrawing a small quantity (20 μl) of the SF suspension from the test chamber and the bubble, after being equilibrated for five min at 37 C, was pulstaed between a maximum radius (r_{max}) of 0.55 mm and a minimum radius (r_{min}) of 0.40 mm for one min. Surface tensions were calculated from the pressure tracings on a strip chart recorder, by a pressure transducer using the Laplace equation: $\Delta P = -2\sigma/r$, in which P, σ and r refer to the pressure drop, surface tension and radius of the spherical bubble, respectively (6).

Chemical analysis. Lipids were extracted from the isolated SF following the standard procedure of Folch et al. (7). The extracted total lipid was quantitated by gravimetry (8) and also following the method of Frings and Dunn (9), using olive oil as the standard. The neutral lipids and phospholipids (PL) from the total lipid were separated by batch chromatography on silicic acid column following the method of Vance and Sweeley (10). Individual PL were separated by single-dimensional thin layer chromatography (TLC) at room temperature on Silica Gel G and was developed by the development system of Mitnick et al. (11). For analytical purposes, the plates were exposed to I_2 vapor and marked. Amounts of individual PL were estimated in the scraped silica gel by phosphorus analysis (12) and computed with an average mol wt. of 775. Neutral lipids also were separated by TLC on Silica Gel G, using the solvent system, petroleum ether/diethyl ether/glacial acetic acid (90:10:1, v/v/v). Cholesterol and unesterified fatty acids were quantitated by the standard methods of Zak (13) and Novak (14), respectively. Cholesterol also was quantitated by gas liquid chromatography (GLC), using cholestenone as the internal standard. Triglyceride was quantitated by glycerol analysis (15), using glycerol trioleate as the standard.

Protein was determined by the modified sodium dodecyl sulfate (SDS)-Lowry procedure using crystallized bovine serum albumin as the standard (16). Nitrogen was analyzed by the method of Lang (17). Hexose and hexosamine were determined by the method of Sheflar (18). Equal amounts of D-mannose and D-galactose served as the hexose standard while D-glucosamine and D-galactosamine for hexosamine. Nucleic acids (DNA and RNA) were extracted by the method of Schneider (19). DNA was determined by the method of Burton (20), using calf thymus DNA as the standard, while RNA was estimated spectrophotometrically following the method of Munro and Fleck (21), utilizing the molar extinction coefficient of RNA at 260 nm. Sialic acid was estimated after acid hydrolysis following the method of Warren (22), and N-acetyl neuraminic acid was used as the standard. All chemical analyses were done in quadruplicate or more.

GLC of the methyl esters of fatty acid moieties of individual lipid classes was done after transesterification (23). GLC was performed with a Hewlett Packard instrument, using a column packed with EGSS-X on chromosorb with heptadecanoate as internal standard. The chromatographic conditions used were injection temperature 250 C, isothermal column temperature 186 C and nitrogen carrier gas at 54 lb/in.^{2}. The column was standardized using fatty acid methyl ester standards (Applied Science Laboratories, State College, PA), and the identification of unknown methyl esters was based upon a comparison of retention times of standards and the unknown. The chromatograms were directly encoded on punch cards, and the relative weight percentage of each methyl ester was calculated by computer integration of the areas under each curve. Fatty acids of PL classes were transesterified and transmethylated according to Metcalfe et al. (24). Fatty acid analysis was done in duplicate.

Enzyme assays. Glucose-6-phosphate phosphohydrolase was assayed following the method of Freedland and Haprer (25); the liberated phosphorus was estimated by either the method of Fiske and Subbarow (26) or Chen et al. (27), using potassium dihydrogen phosphate as the standard. Acid phosphatase and alkaline phosphatase (AKP) activities were assayed as described by Bergmeyer (28), using p-nitrophenyl phosphate as the substrate, and the liberated p-nitrophenol was mesaured at 419 nm. ($Na^+ + K^+$)-dependent ATPase estimation was carried out according to Quigley and Gotterer (29), and the enzyme activity was obtained by subtracting ouabain insensitive ATPase from the total ATPase. 5′-Nucleotidase was assayed according to Heppel and Hilmoe (30). β-N-acetyl glucosaminidase was estimated following the method of Sugden and Lazou (31). NADPH-cytochrome C reductase was estimated by the method of Mazel (32). Reduction of cytochrome P450 was followed spectrophotometrically at 550 nm and an extinction coefficient of 19.1 mM^{-1}/cm of the reduced form was utilized to calculate the enzyme activity. Succinic dehydrogenase was assayed following the method of Kun and Abood (33). For all enzyme analyses, SF samples were suspended in 0.1% Triton X-100 before the enzyme assay.

Isolation of SF-associated apoproteins. SF is dialyzed against 5 mM ethylenediamine tetraacetic acid (EDTA) in water for 24 hr, and the dialysate was lyophilized. The lyophilized material was delipidated according to Scanu et al. (34). Ethanol/ether (1:3, v/v) at −20 C was added to the dry powder and the lipids extracted overnight at −20 C. The protein precipitated as a fine white powder, which was pelleted by centrifugation at 10,000 × g for 30 min. The precipitate was reextracted twice more, first with 1:3 (v/v) ethanol/ether for four hr, followed by ether for four hr at −20 C. Both the supernatants and the precipitates were analyzed for their PL and protein contents. The precipitate was dried under a N_2 stream and suspended in buffer B (0.05 M sodium borate, pH 9.0, containing 0.1% SDS). The suspension was sonicated with a probe-type sonicator (MSE) with moderate power setting (12 amplitude microns) in an ice-water environment for 10 min and then centrifuted at 40,000 × g for one hr. The clear supernatant was used for sephadex gel filtration chromatography and polyacrylamide gel electrophoresis.

Sephadex gel filtration chromatography. Proteins were chromatographed on columns of sephadex G-200 (40–120 μm dry bead diameter, Pharmacia, Piscataway, NJ),

equilibrated with buffer B using a 90 × 2.5 cm (i.d.) column having a bed volume of 450 ml and a void volume of 150 ml as determined with blue dextran. They were eluted with buffer B at a flow rate of 25 ml/hr, monitored by UV absorption at 280 nm, and a 5 ml fraction of each was collected.

Polyacrylamide gel electrophoresis. Polyacryamide disc gel electrophoresis of the apoprotein was done in 10% acrylamide gels, uniformly in buffer B, essentially following the method of Weber and Osborn (35) at a constant current of 5 mA per gel. About 100 μg protein in 100 μl vol was applied in 0.2% SDS solution with 0.05% bromophenol blue as the tracking dye and 5 μl of 2-mercaptoethanol. After the electrophoresis was completed, the gels were treated with 10% trichloroacetic acid for 30 min, stained with 0.25% Coomassie blue in 50% methanol for four hr and destained overnight by diffusion in 10% acetic acid in water.

Electron microscopy. Pellets of SF were fixed for one hr with 3% glutaryldehyde in 0.1 M sodium phosphate buffer, pH 7.4, at room temperature and then post-fixed in 1% OsO_4 in the same buffer for one hr at 4 C, followed by several changes of 100% alcohol at room temperature. Pellets were infiltrated with a 1:1 (v/v) mixture of epon and propylene oxide at room temperature with constant shaking for one hr, followed by shaking overnight in 4:1 (v/v) mixture of the same. They subsequently were embedded in pure epon, and ultrathin sections were cut in a LKB Loba ultramicrotome, stained with lead citrate and uranyl acetate and finally examined with a Jeol 1200 XE electron microscope. The EM procedure was based on the method of Collet (36).

Immunological analysis. Antibodies directed against the purified surface-active material were obtained from New Zealand white rabbits by injecting intramuscularly at multiple sites in the hindquarters and footpads of rabbits 10 mg of SF mixed with 2 ml of complete Freund's adjuvant. The animals were reinjected with 1 mg of SF without adjuvant, one, two and three wk after the initial injection. If needed, the animals were fortified with 0.2 mg protein injected intravenously. Blood was collected from the ear vein four wk after the first injection and then weekly while the antibody titers remained high. The antiserum was separated by centrifugation and adsorbed for 60 min at 37 C against pooled human serum by mixing one drop of human serum with 1 ml of rabbit antiserum to remove antibody activity that reacted with this substance. The resulting precipitate was removed by centrifugation at 5,000 × g for 20 min, and the adsorption was repeated by mixing one drop of human serum in a 1:10 dilution (serum/normal saline, v/v) with 1 ml of antiserum incubated at 37 C for 60 min to separate the precipitate. The adsorption was repeated with human serum in a 1:100 dilution until no precipitate was visible. The antigen, suspended in solution containing 0.2% SDS, was studied using a double diffusion precipitation reaction in 0.75% agarose gels (Biorad Laboratories, Richmond, CA) following the procedure of Clausen (37). The gels were swollen in 0.05 M sodium barbital buffer, pH 8.0, containing 0.05% of SDS, and the reactions were carried out at room temperature in a moist atmosphere for 48 hr. The precipitation patterns were clearly visible after staining with Coomassie blue and then photographed with suitable backlighting.

Unless otherwise noted, analytical reagent-grade chemicals were used in all the experiments without further purification. Solvents were redistilled before use. The standards used in each of the analyses were specified by the manufacturers as being chromatographically homogeneous.

RESULTS

As reported in this paper, a SF fraction from human lung lavage fluid collected at autopsy is isolated and purified free of other subcellular contaminants such as mitochondria, lysosomes, endoplasmic reticulum and type-II cell plasma membranes. A low-power EM photomicrograph of the isolated material (Fig. 1) shows a considerable degree of purity with no apparent contamination. The fraction consists of membranous vesicles, lamellar structures and sheets of membranous materials. Vesicles with well-preserved limiting membranes and inclusion bodies are observed.

The chemical compositions of SF are given in Table 1. It is comprised almost entirely of lipid and protein with

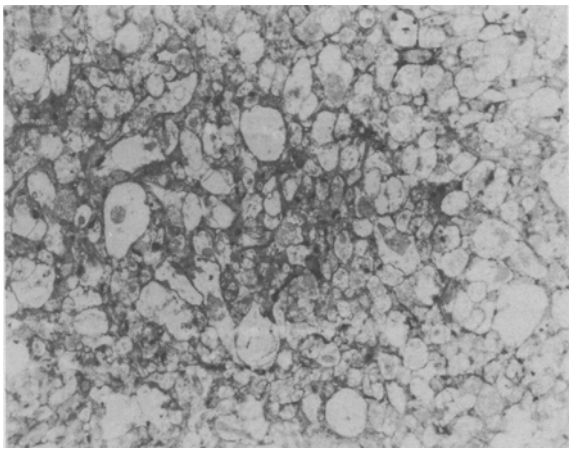

FIG. 1. Electron micrograph of surfactant isolated and gradient purified from adult human lung lavage (×3000).

TABLE 1

Chemical Composition of Human Pulmonary Surfactant

Chemical constitutent	mg/ml	% of dry wt
Dry wt (gravimetry)	10.90 ± 0.003	—
Protein	2.185 ± 0.012	20.04
Total lipid	7.99 ± 0.11	73.30
DNA	0.022 ± 0.002	0.20
RNA	0.061 ± 0.0003	0.55
Phosphorus	0.218 ± 0.006	2.0
Total sialic acid	0.0573 ± 0.0008	0.52
Hexose	0.174 ± 0.011	1.59
Hexosamine	0.046 ± 0.004	0.42
Nitrogen	0.379 ± 0.009	3.47

Results are mean ± SD of four to six observations. Whole lungs of either sex weighing between 700–1000 g were lavaged with 3 l of chilled buffer, with the average recovery of 60–70% of lavage fluid (ca. 2 l), which was processed for purification of SF. The isolated material finally was suspended in 5 ml of the buffer, and an aliquot was taken for determination of dry mass by gravimetry and also for analyzing each of the chemical constituents reported here and in the subsequent tables.

only small amounts of hexose, hexosamine, nucleic acids and sialic acid. The total lipid constitutes 73.34% of the SF dry wt., while the protein present is about 20%. About 2% phosphorus and 3.5% nitrogen also are present in the material. Lipid compositions of the isolated fraction (Table 2) show that PL constitutes more than 78% of the total lipid weight. Among the individual PL classes, phosphatidylcholine (PC) constitutes about 69% of total PL and phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are 8% and 11%, respectively. Lysophosphatidylcholine, phosphatidylinositol (PI) and sphingomyelin constitute relatively minor components of the total PL. Six neutral lipid fractions also can be identified by chemical analyses. These are cholesterol, cholesteryl ester, unesterified fatty acids and mono-, di- and triacylglycerol. Mono- and diacylglycerols in extremely small amounts also are detected by GLC constituting less than 1% of the total lipid. A seventh component migrating with mobility less than cholesterol has been isolated by TLC, but its identity cannot be established. A negligible amount of lipid-bound sialic acid also is present in the form of gangliosides, but cannot be quantitated by conventional chemical analysis.

Fatty acid compositions of the isolated PL classes are shown in Table 3. The predominant fatty acid of PC and PG is palmitic acid (16:0), while oleic acid (18:1) is the

TABLE 2

Lipid Composition of Human Pulmonary Surfactant

Lipid class	mg/ml of surfactant	% of total lipid	% of neutral or phospholipid	% of dry wt
Total lipid	7.99 ± 0.11		—	73.34
Total neutral lipid	1.88 ± 0.024	22.44	—	—
Cholesterol	0.746 ± 0.007	9.56	42.60	6.86
Cholesteryl ester	0.227 ± 0.009	2.91	12.96	2.07
Unesterified fatty acid	0.179 ± 0.001	2.29	10.20	1.63
Triacylglycerol	0.599 ± 0.005	7.69	34.22	5.47
Diacylglycerol	—	<1.0	—	—
Monoacylglycerol	—	<1.0	—	—
Total phospholipid	6.11 ± 0.145	78.44	—	56.13
Phosphatidylcholine	4.199 ± 0.032	53.80	68.58	38.53
Phosphatidylethanolamine	0.516 ± 0.009	6.62	8.43	4.73
Phosphatidylinositol	0.154 ± 0.006	1.97	2.51	1.41
Phosphatidylglycerol	0.699 ± 0.011	3.95	11.40	6.40
Sphingomyelin	0.128 ± 0.006	1.63	2.07	1.17
Lysophosphatidylcholine	0.121 ± 0.009	1.55	1.97	1.11
Phosphatidylserine	—	<1.0	—	—
Lipid-bound sialic acid	—	<1.0	—	—

Results are mean ± SD of four to six observations. See Table 1 for explanation of the expression mg/ml and % of dry wt. Dry mass per ml of the SF sample is found to be 10.9 mg as shown in Table 1.

TABLE 3

Fatty Acid Composition of Individual Phospholipid Classes of Human Pulmonary Surfactant

	% wt			
Fatty acid	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylglycerol	Phosphatidylinositol
14:0	4.1	0.5	0.6	0.6
14:1	1.6	—	0.9	—
14:2	0.3	2.6	—	—
16:0	62.9	15.8	49.6	23.0
16:1	8.2	4.0	4.6	4.3
16:2	0.2	0.4	0.4	2.2
18:0	2.9	9.5	5.3	18.1
18:1	16.8	47.0	33.8	31.0
18:2	1.3	8.1	2.7	3.9
18:3	0.6	1.2	0.6	0.6
18:4	0.5	1.1	0.3	—
20:0	—	—	—	0.6
20:4	0.4	4.7	0.9	7.5
20:5	—	0.7	—	—
22:5	—	0.5	0.3	0.5
22:6	—	2.0	—	4.3
Unknown	0.6	1.9	—	0.4
Total saturated	69.9	25.8	55.5	42.3
Total unsaturated	29.5	72.3	44.5	54.3
Saturated/unsaturated	2.36	0.35	1.24	0.77

Results are mean of two independent observations.

main fatty acid present in the PE and PI fractions. Interestingly, 16:0 and 18:1 are quantitatively the next highest fatty acid components of PE/PI and PC/PG, respectively. A large variety of fatty acids differing in carbon number, chain length and number of double bonds are present in these PL fractions, ranging from 14:0 to 22:6. Saturated fatty acids consist of about 69.9 and 55.5% weight composition of total fatty acids for the two most important PL classes of surfactant, i.e. PC and PG, while PE and PI are more unsaturated.

Enzymatic analysis of SF indicates that it contains a very high specific activity of AKP (Table 4), but acid phosphatase activity is almost nonexistent. A number of subcellular marker enzymes also are assayed in the surfactant. There are negligible activities of lysosomal hydrolases such as β-N-acetyl glucosaminidase and microsomal markers like glucose-6-phosphatase and NADPH-cytochrome C reductase. Mitochondrial-specific succinic dehydrogenase and plasma membrane associated enzymes like $(Na^+ + K^+)$-dependent ATPase and 5′-Nucleotidase are not detected. These results suggest that our SF-enriched fraction apparently is not contaminated by endoplasmic reticulum, mitochondria, lysosomes or type-II cell plasma membranes and substantiate the EM observations.

Isolation of SF material is monitored systematically at each step of centrifugation by using PL/protein ratio, AKP-specific activity and surface activity of the isolated material as indications of purity. As shown in Table 5, the isolated surfactant from the stepwise gradient centrifugation step (75,000 × g sucrose density) represents considerable purity (× 196) based on the PL/protein ratio and 450-fold purity based on the AKP-specific activity compared with the initial lung lavage fluid. Additional centrifugation in a discontinuous sucrose density gradient does not significantly alter the ratio of PL to protein or AKP specific activity and, therefore, does not increase the purity but actually results in more than 60% loss of the purified sample and thus is avoided.

During delipidation, the substance precipitating in the ether/ethanol solution contains about 10% of the recovered phosphorus and more than 90% of the recovered proteins. The supernatant contains the remaining phosphorus but little or no amount of protein. The polyacrylamide gel electrophoresis in 10% acrylamide of the total protein is shown in Figure 2. There are three major components stained with Coomassie blue and one or two minor bands. The molecular weight assignments are based on comparison of the mobilities of protein standards with those recovered from the SF. The major components show molecular weight of about 68,000, 34,000 and 10,000 daltons. Sometimes, a high molecular weight material (>100,000) also is seen at the top of the gel, representing aggregated protein and probably not associated with SF. Gel filtration chromatography of these

TABLE 4

Specific Activities of Some Hydrolases and Other Subcellular Marker Enzymes in Human Pulmonary Surfactant

Enzymes	Specific activity (nmol/min/mg protein)
Alkaline phosphatase	506.7
Acid phosphatase	3.25
Glucose-6-phosphate phosphohydrolase	9.55
β-N-acetyl glucosaminidase	N.D.
NADPH-cytochrome C reductase	42.84
$(Na^+ + K^+)$-dependent adenosine triphosphate phosphohydrolase	N.D.
5′-Nucleotidase	18.70
Succinic dehydrogenase	N.D.

Results are means of four to six observations.
N.D., not detectable.

TABLE 5

Purification of Surfactant from Human Bronchoalveola Lavage Fluid

Isolation step	Surface tension at 1 min pulsation (mN/m)		Phospholipid/protein (mg/mg)	Alkaline phosphatase (nmol/min/mg protein)	Enrichment (-fold)		
	r_{max}	r_{min}			Surface tension at r_{min}	Phospholipid/ protein	Alkaline phosphatase
Crude bronchoalveolar lavage	72	27	0.27	2.06			
160 × g supernatant	58	21	0.27	1.86			
40,000 × g pellet	55	10	52.74	820.9	2.7	195.33	398.49
18,000 sucrose density gradient	45	8	53.15	801.21	3.3	196.85	388.93
Purified surfactant (75,000 × g sucrose stepwise density gradient)							
Fraction I	14	0.8	52.88	927.74	33.75	195.85	450.35
Fraction II	17	1.0	42.3	617.54	27	156.66	299.77
75,000 × g pellet	63	31	0.29	2.49			

Results are means of three observations. Isolation steps are carried out with 100 ml of initial lavage fluid.

proteins using Sephadex G-200 results in two separate fractions (Fig. 3). The first fraction contains mostly 68 kDa protein, which has identical electrophoretic mobility with serum albumin, while the second fraction eluted from the column contains 34 kDa and 10 kDa proteins that represent non-serum components. They also show the maximum surface activity and are consequently the proteins of particular interest in this study.

The results of a double diffusion immunoprecipitation in 0.75% agarose gel are shown in Figure 4A. The center well contains the antiserum directed against the whole surfactant that has been adsorbed against pooled human serum. The outer wells contain (1) purified and delipidated whole human lung lavage surface-active material, (2) peak II of Sephadex G-200 gel filtration chromatography of the isolated and delipidated SF and (3) peak I of the same. Both, whole SF and the material in the second Sephadex fraction, give one strong precipitation line with the antiserum. Figure 4B shows the immuneprecipitin reaction when two different dilutions of the antigen are used. Wells 1 and 3 contain 1:3 dilution of the surface-active material to normal saline while wells 2 and 4 represent 1:4 dilution

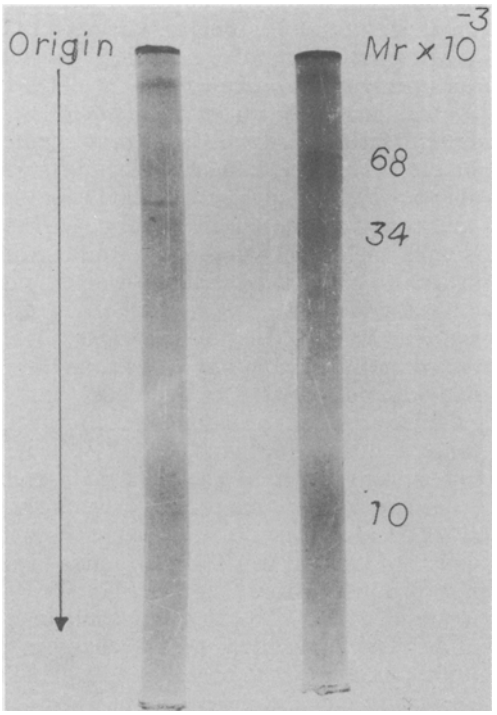

FIG. 2. Analysis of surfactant associated proteins by SDS-polyacrylamide gel electrophoresis. Electrophoresis is done in 10% acrylamide gels, using 0.05 M sodium borate buffer, pH 9.0, containing 0.1% SDS. The two gels indicate the independently isolated surface-active materials from bronchoalveolar lavage fluid of two different sets of lungs. The isolated materials are lyophilized and delipidated before electrophoresis.

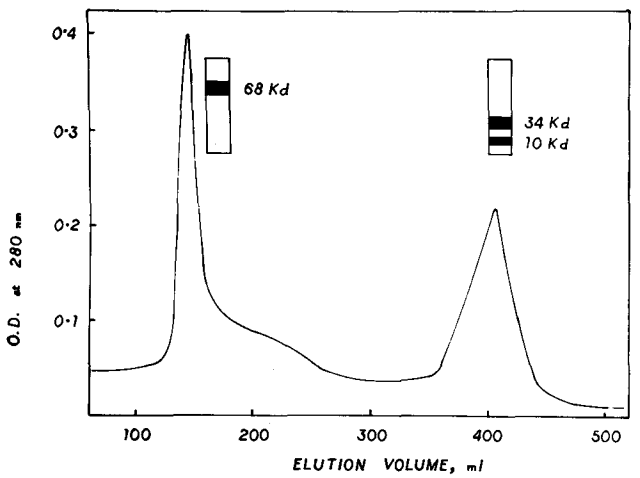

FIG. 3. Results of Sephadex G-200 gel filtration and polyacrylamide gel electrophoresis of proteins from human lung surface-active material. Gel filtration studies are carried out twice, and a representative result is shown.

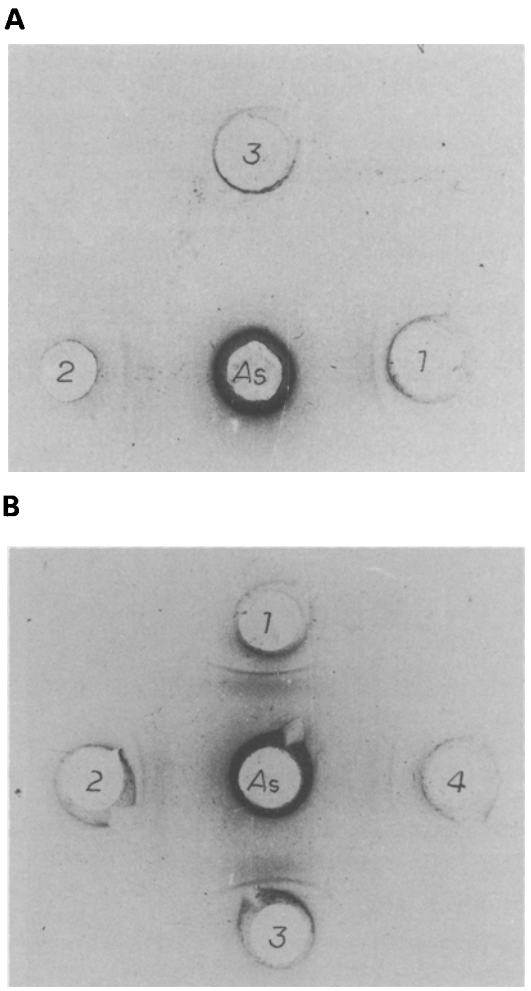

FIG. 4. (A) Double-diffusion immunoprecipitation in 0.75% agarose gel of lung surface-active material. (1) Purified surfactant from human lung lavage, (2) Peak II of the gel filtration chromatography, and (3) Peak I of the same. Center well contains antiserum against surfactant extensively adsorbed against pooled human serum. (B) Immune precipitin reaction with two different dilutions of the surface-active material. Center well contains antiserum against surfactant extensively adsorbed against human serum. 1 and 3 contain 1:3 (SF to normal saline) diluted surface-active material, whereas, 2 and 4 contain 1:4 dilution of the same. The surface-active material used is that purified through the 75,000 × g stepwise sucrose density gradient step (Fraction-I).

of the same. No diffusion of the pulmonary surface-active material takes place in agarose unless a dispersing agent such as SDS in 0.05% concentration is added to the gel.

DISCUSSION

In this work, SF from human lung lavage fluid have been isolated, and their biochemical, morphological and immunological compositions are characterized. Some investigators have developed a method to isolate lung SF from tissue homogenates (38,39). However, the risk of contamination using these methods is much higher because the lung tissue contains numerous cell types (40). Our method involves low-speed centrifugation for removal of cellular debris, followed by high-speed centrifugation and final purification by gradient centrifugation. The major advantage of the procedure, apart from the use of a single-step sucrose gradient in an ultracentrifuge, is that the centrifugation time is much shorter than most of the methods published before. Such combinations of differential and density gradient centrifugations yield SF fractions that are far more enriched based on phospholipid to protein ratio, AKP specific activity and surface activity than the crude lavage fluid. Our EM and marker enzyme data also establish the purity of the material, free of other lung subcellular contaminants. Presence of high concentrations of AKP in the isolated material leads us to believe that insoluble AKP probably is secreted from the lung lamellar bodies, and they can be used as potential markers for studies on synthesis and secretion of pulmonary SF. Tubular myelin-like figures, as observed by others in the ultrastructural studies of SF, are absent in our material (41,42) possibly because our isolation buffer did not contain Ca_{2+}, which tends to stabilize ordered lattice structures.

The chemical composition of pulmonary surface active material has been investigated in experimental animals in several laboratories (43) and there is general agreement that it is rich in PL, especially dipalmitoyl phosphatidylcholine (DPPC). Whether the protein associated with it qualifies it as a lipoprotein has been debated. Even after delipidation, the protein precipitated in ethanol/ether still retains 10% phospholipids. In the presence of PL, the surfactant-associated apoproteins migrate as a broad, blurred band in our electrophoretic experiments. PL analyses of the isolated SF show that the major components are either zwitterionic (PC) or acidic (PG), and, therefore, the firm association of PL-protein may occur through divalent cation linkage, hydrophobic interactions or both. Preponderance of the saturated fatty acid, palmitic acid, in these two PL of SF is confirmed by GLC analysis. Substantial amounts of monoenoic PL and neutral lipids also are present in SF. The functions of these lipids are not certain, but they do lower the temperature of an endothermic phase change or transition, called melting in synthetic lipid mixtures containing DPPC, which may be essential to the velocity of adsorption rates that are required in the alveolar lining.

The isolated material also contains two nonserum proteins of 34 and 10 kDa, respectively, which give a partial identity reaction with antiserum directed against the whole SF. Recently, there have been reports of the presence of a very low molecular weight protein, 5–6 kDa, in the lung surfactant system (44–46), which also is found to be chloroform-soluble. However, our method of isolation, delipidation and protein analysis by gel electrophoresis may exclude the presence of this extremely hydrophobic protein. SF apoproteins apparently function by promoting the formation of a surface active monolayer from bilayer lipids through a lipid-lipid interaction (46). However, the precise mechanism by which they influence lipid structures and/or mobility is not known. The immuno properties of these proteins will be used as a basis for a quantitative assay of surface-active material in small lung samples obtained by biopsy in further experiments to investigate the metabolism of SF and in samples of tracheal or amniotic fluid. The ultimate goal of this useful technique will be the prenatal assessment of lung maturity. Our method of isolation also can be used in large-scale isolation of SF required for therapeutic use in RDS of the neonates.

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REFERENCES

1. Farrell, P.M., and Avery, M.E. (1975) *Am. Rev. Respir. Dis. 111,* 657–688.
2. Goerke, J. (1974) *Biochim. Biophys. Acta 344,* 241–261.
3. Askin, F.B., and Kuhn, C. (1971) *Lab. Invest. 25,* 260–268.
4. Avery, M.E., and Mead, J. (1959) *Am. J. Dis. Child. 97,* 517–523.
5. Enhorning, G. (1977) *J. Appl. Physiol. 43,* 198–203.
6. Possmayer, F., Yu, S.H., Weber, J.M., and Harding, P.G.R. (1984) *Can. J. Biochem. Cell Biol. 62,* 1121–1133.
7. Folch, J., Less, M., and Sloane Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497–509.
8. Sperry, W.M. (1963) in *Standard Methods of Clinical Chemistry* (Seligson, D., ed.) Vol. 4, pp. 173–182, Academic Press, New York.
9. Frings, C.S., and Dunn, R.T. (1970) *Am. J. Clin. Pathol. 53,* 89–91.
10. Vance, D.E., and Sweeley, C.C. (1967) *J. Lipid Res. 8,* 621–630.
11. Mitnick, M.A., DeMarco, B., and Gibbons, J.M. (1980) *Clin. Chem. 26,* 277–281.
12. Bartlett, G.R. (1959) *J. Biol. Chem. 234,* 466–468.
13. Zak, B. (1957) *Am. J. Clin. Pathol. 27,* 583–588.
14. Novak, M. (1965) *J. Lipid Res. 6,* 431–435.
15. Van Handel, E., and Zilversmit, D.B. (1957) *J. Lab. Clin. Med. 50,* 152–157.
16. Lees, M.B., and Paxman, S. (1972) *Anal. Biochem. 47,* 184–192.
17. Lang, C.A. (1958) *Anal. Chem. 30,* 1692–1694.
18. Sheflar, M.R. (1966) in *Progress in Clinical Pathology, Glycoproteins* (Stefanini, M., ed.) Vol. 1, pp. 423–457, Grune and Stratton, New York.
19. Schneider, W.C. (1945) *J. Biol. Chem. 161,* 293–303.
20. Burton, K. (1956) *Biochem. J. 62,* 315–323.
21. Munro, H.N., and Fleck, A. (1966) in *Methods of Biochemical Analysis* (Glick, D., ed.) Vol. 14, pp. 113–176, Interscience Publishers, New York.
22. Warren, L. (1959) *J. Biol. Chem. 234,* 1971–1975.
23. Grenier, G., Marier, J.P., and Beaumont, G. (1979) *Can. J. Bot. 57,* 1015–1020.
24. Metcalfe, L.C., Schusitz, A.A., and Pelka, J.R. (1966) *Anal. Chem. 38,* 514–515.
25. Freedland, R.A., and Harper, A.E. (1959) *J. Biol. Chem. 234,* 1350–1354.
26. Fiske, C.H., and Subbarow, Y. (1925) *J. Biol. Chem. 66,* 375–400.
27. Chen, P.S., Teribara, T.Y., and Warner, H. (1956) *Anal. Chem. 28,* 1756–1758.
28. Bergmeyer, M.V.C. (1963) in *Methods of Enzymatic Analysis,* Vol. 1, pp. 783–789, Academic Press, New York.
29. Quigley, J.P., and Gotterer, G.S. (1969) *Biochim. Biophys. Acta 173,* 456–463.

30. Heppel, L.A., and Hilmoe, R.J. (1955) in *Methods in Enzymology* (Colowick, S.P., and Kaplan, N.O., eds.) Vol. 2, pp. 546-550, Academic Press, New York.
31. Sugden, P.H., and Lazou, A. (1984) *Cardiovasc. Res. 18,* 483-485.
32. Mazel, P. (1971) in *Fundamentals of Drug Metabolism and Drug Disposition* (La Du, B.N., ed.) pp. 546-575, Williams and Wilkins, Baltimore, MD.
33. Kun, E., and Abood, L.G. (1949) *Science 109,* 144-146.
34. Scanu, A., Pollard, H., Hirz, R., and Kothary, K. (1969) *Proc. Natl. Acad. Sci. USA 62,* 171-178.
35. Weber, K., and Osborn, M. (1969) *J. Biol. Chem. 244,* 4406-4412.
36. Collet, A.J. (1979) *J. Histochem. Cytochem. 27,* 989-996.
37. Clausen, J. (1969) in *Immunochemical Techniques for the Identification and Estimation of Macromolecules,* pp. 399-572, North Holland Publishing Co., Amsterdam and London.
38. Gil, J., and Reiss, O.K. (1973) *J. Cell Biol. 58,* 152-171.
39. Frosolono, M.F., Charmis, B.L., Pawlowski, R., and Slivka, S. (1970) *J. Lipid Res. 11,* 439-457.
40. Sorokin, S.P. (1970) *AEC Symposium Service 21,* 3.
41. Boudreau, J., Beaudoin, A.R., and Nadeau, D. (1983) *Can. J. Biochem. Cell Biol. 61,* 231-239.
42. Ng, V.L., Herndon, V.L., Mendelson, C.R., and Snyder, J.M. (1983) *Biochim. Biophys. Acta 754,* 218-226.
43. Clements, J.A. (1970) *Am. Rev. Respir. Dis. 101,* 984-990.
44. Phizackerley, P.J.R., Town, M.H., and Newman, G.E. (1983) *Biochem. J. 183,* 731-736.
45. Claypool, W.D., Wang, D.L., Chander, A., and Fisher, A.B. (1984) *J. Clin. Invest. 74,* 677-684.
46. Yu, S.H., and Possmayer, F. (1986) *Biochem. J. 236,* 85-89.

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Promotion of Iron-induced Rat Liver Microsomal Lipid Peroxidation by Copper

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Although copper has been demonstrated to promote lipid peroxidation in a number of systems, the mechanisms involved have not been fully defined. In this study, the role of copper in modifying lipid peroxidation has been explored in rat hepatic microsomes. In an in vitro system containing reduced glutathione (GSH, 200 μM) and Tris buffer, pH 7.4, cupric sulfate (1–50 μM) potentiated lipid peroxidation induced by ferrous sulfate (10 μM) but was unable to elicit peroxidation in the absence of iron. Higher levels of cupric sulfate (100 μM or greater) were inhibitory. The nature as well as the extent of the peroxidative response of microsomes to cupric sulfate were dependent on glutathione levels in addition to those of iron. Cupric sulfate (100 μM) strongly potentiated ferrous ion-induced lipid peroxidation in the presence of 400–800 μM GSH, while it inhibited peroxidation at lower levels of GSH (0–200 μM) and did not affect ferrous ion-induced peroxidation with glutathione levels of 3–10 mM.

The potentiating effect of copper on ferrous ion-induced lipid peroxidation was further explored by investigating: (1) potential GSH-mediated reduction of cupric ions; (2) potential copper/GSH-mediated reduction of ferric ions (formed by oxidation during incubation); and (3) possible promotion of propagation reactions by copper/GSH. Our results indicate that cupric ions are reduced by GSH and thus are converted from an inhibitor to an enhancer of iron-induced lipid peroxidation. Cuprous ions appear to potentiate lipid peroxidation by reduction of ferric ions, rather than by promoting propagation reactions. Iron (in a specific Fe^{+2}/Fe^{+3} ratio) is then an effective promoter of initiation reactions.
Lipids 23, 559–563 (1988).

Iron is an active agent throughout the reaction sequences involved in microsomal lipid peroxidation. Specific iron chelates promote the initiation phase of peroxidation, while other iron chelates are thought to act as propagators (1,2). The role of copper in lipid peroxidation is less defined. Hochstein et al. (3) demonstrated the formation of fluorescent chromolipids in human erythrocytes exposed to cupric sulfate. They suggested that cupric ions are reduced by membranous sulfhydryl groups (regenerated by reduced glutathione [GSH]) and subsequently react with molecular oxygen to yield superoxide anions which in turn promote peroxidation. Ding and Chan (4) described copper catalyzed lipid peroxidation in erythrocyte membranes following generation of membranous lipid hydroperoxides by exposure to ultraviolet light. Hydroperoxides were essential for this peroxidative response to copper, suggesting that copper promotes the degradation of lipid hydroperoxide and thus promotes the propagation phase of the peroxidation mechanisms.

In contrast with these findings, Wittig and Steffen reported that cupric ions inhibit NADPH-dependent rat liver lipid peroxidation (5). The inhibition of microsomal lipid peroxidation by copper paralleled a similar effect of 100,000 × g supernatant (containing equivalent concentrations of copper).

As part of an effort to define agents that act as initiators and/or propagators of lipid peroxidation, we have explored the effect of copper on basal and ferrous ion-initiated peroxidative responses.

EXPERIMENTAL

Materials. ADP, bathocuproine, cytochrome c (Type III from horse heart), GSH, 2-thiobarbituric acid (TBA) and xanthine were obtained from Sigma Chemical Co. (St. Louis, MO). Ferric chloride and ferrous sulfate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Cupric sulfate was purchased from Merck and Co. (Rahway, NJ).

Enzymes. Bovine erythrocyte superoxide dismutase (SOD) (EC 1.15.1.1) was obtained from Miles Biochemical (Elkart, IN). Xanthine oxidase (EC 1.2.3.2) was purchased from Sigma Chemical Co.

Preparation of microsomes. Liver microsomes were prepared from 20 g of tissue from a 10% homogenate in 0.15 M KCl, 10 mM Tris buffer, pH 7.4, by performing differential centrifugation. The pellet obtained by centrifuging the 20,000 × g supernatant at 100,000 × g was resuspended in homogenization buffer (5–10 mg protein/ml) and assayed for protein content (6).

Preparation of oxidized and control phospholipid liposomes. Phospholipid hydroperoxides were prepared from egg phosphatidylcholine (Type III E, Sigma Chemical Co.) using soybean lipoxygenase-1 and incubating one hr at pH 9.0 in the presence of 10 mM deoxycholic acid as described by Eskola and Laakso (7). The extent of formation of phospholipid hydroperoxide was monitored by U.V. absorbance (232–235 nm) using a molar coefficient of 27,000 M^{-1} cm^{-1} (8). Chloroform/methanol solutions of oxidized and unoxidized phosphatidylcholine were combined such that 10% of the phospholipid was oxidized. Following evaporation of organic solvents, phospholipid liposomes were prepared by sonication in water to a concentration of 1 mM phosphatidylcholine. Control phospholipid liposomes were prepared in the same manner omitting the phospholipid hydroperoxide and substituting unoxidized phospholipid.

In one experiment, liposomes were prepared from the total (neutral plus polar) lipids of liver microsomes by sonication at their original concentration in homogenization buffer. An Aminco-Bowman spectrofluorometer was used with a relative intensity of 1.0 being defined as a reading of 10 on sensitivity setting 10. Standard solutions yielded relative intensities of 0.9, 1.7, 3.25 and 6.1 for 0.5,

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Abbreviations: ADP, adenosine 5′-diphosphate; GSH, reduced glutathione; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid.

1.0, 2.0 and 4.0 nmol of malondialdehyde. More intense samples were diluted 10:1 in butanol before reading.

Other assays. Cuprous copper was detected by measurement of complex formation with bathocuproine (11). Ferrous iron was detected by measurement of reactivity with 1,10-phenanthroline (12).

RESULTS

Cupric sulfate (1–50 μM) potentiated ferrous ion-promoted lipid peroxidation in a system consisting of rat liver microsomes in 100 mM Tris buffer, pH 7.4, containing 200 μM GSH and ferrous sulfate (10 μM) (Fig. 1). However, copper did not induce peroxidation in the absence of iron (Fig. 1). Higher levels of copper (100, 200 μM) inhibited iron-induced lipid peroxidation. A temporal study of ferrous ion-induced lipid peroxidation in the presence and absence of copper/GSH showed the potentiation of peroxidation by 10 μM copper developed after a lag period during which time the peroxidative response to iron alone was rapid (Fig. 2). In the presence of copper/GSH, iron-induced peroxidation continued for a longer period before plateauing. In these initial investigations, adenosine 5′-diphosphate (ADP) (1 mM) was included in reaction mixtures to promote iron-induced peroxidation (1). We determined that in our system, the effect of ADP was a minor (less than 15%) enhancement of both the iron and copper/iron peroxidative responses and did not add ADP in subsequent investigations except where particularly noted. GSH was included in reaction mixtures to facilitate possible redox cycling reactions (3). The dependency of the peroxidative response to copper (in the presence of iron) on GSH is demonstrated in Figure 3. Cupric sulfate was ineffective in the absence of GSH and higher levels of copper (100 μM) inhibited iron-induced peroxidation. At glutathione levels of 400–800 μM, copper-promoted potentiation of ferrous ion-elicited lipid peroxidation was optimal, and higher levels of copper (100 μM) now strongly enhanced the peroxidative response. Cupric sulfate was ineffective, however, in modifying peroxidative responses to iron in the presence of 3–10 mM GSH.

The peroxidative response of microsomes to copper/iron was tested in a 5 mM phosphate buffer (pH 7.4) to investigate possible artifacts due to the use of Tris buffer. Initial results indicated that copper was ineffective in phosphate buffer. Chelation of copper with ADP (10 × concentration in an aqueous solution) before its addition to reaction mixtures resulted in potentiation of iron-induced peroxidation comparable to that seen in Tris buffer (with or without ADP) (data not shown). A similar chelation of iron with ADP did not allow copper induced potentiation of lipid peroxidation in phosphate buffer.

Reduction of cupric ions to the cuprous form by glutathione (400 μM) was demonstrated by measuring complex formation with bathocuproine (Fig. 4) and thus conceivably is involved in copper-promoted peroxidation.

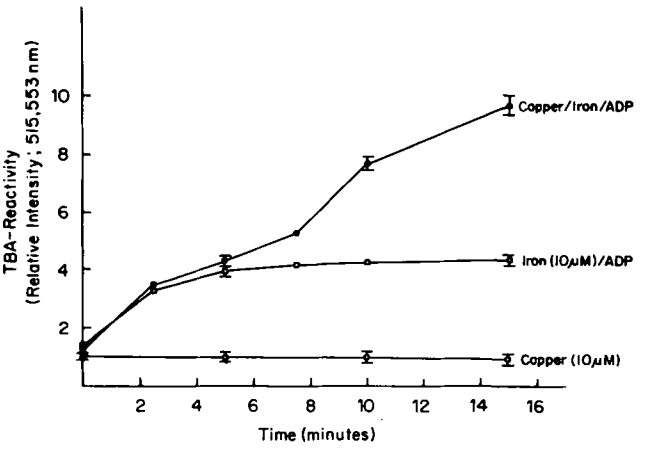

FIG. 2. The time dependency of copper/iron-induced lipid peroxidation. Rat liver microsomes were exposed to cupric sulfate (10 μM) (○), to ferrous sulfate (10 μM) (□) or to the combination of cupric sulfate and ferrous sulfate (same concentration as above) (●) for the indicated duration in the presence of glutathione (200 μM), ADP (1 mM) and 100 mM Tris buffer (pH 7.4) at 37 C. Lipid peroxidation was assessed by a spectrofluorometric measurement of TBA-reactivity. Brackets denote the SEM of four observations.

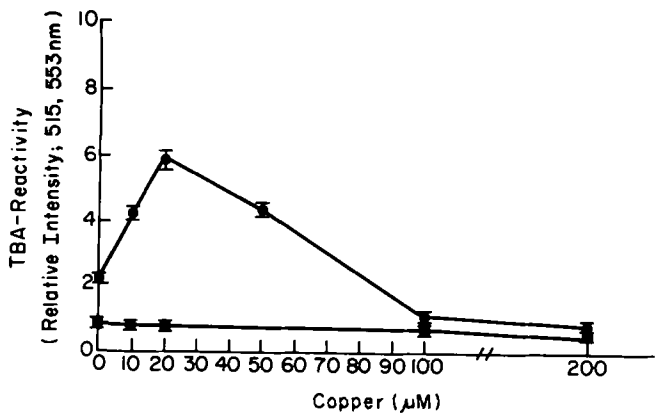

FIG. 1. The effects of copper on basal and iron-promoted microsomal lipid peroxidation. Rat liver microsomes were exposed to the indicated concentration of copper for 15 min at 37 C in the presence of 200 μM GSH in 100 mM Tris buffer (pH 7.4) and (■) no additional agent or (●) ferrous sulfate (10 μM). Lipid peroxidation then was assessed by spectrofluorometric measurement of TBA-reactivity. Brackets denote the SEM of four observations.

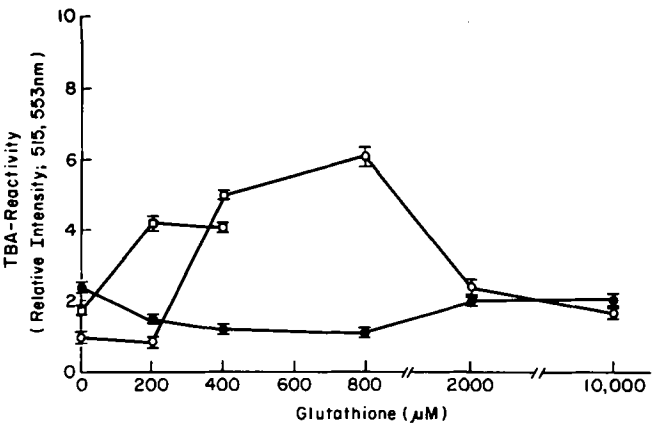

FIG. 3. The GSH dependency of cupric ion-induced lipid peroxidation. Rat liver microsomes were exposed to ferrous sulfate (10 μM) and either no additional metals (●) or to cupric sulfate (10 μM) (□) or cupric sulfate (100 μM) (○). GSH was included in reaction mixtures at the indicated concentration. Lipid peroxidation was assessed as described above. Brackets denote the SEM of four observations.

A possible mechanism for cupric sulfate/GSH-promoted peroxidation is that iron, oxidized to the ferric form during the course of incubation, is reduced by cuprous ion (a thermodynamically feasible process) and may exert an amplified peroxidative response. In microsome-free incubation media, rapid oxidation of ferrous ion was demonstrated (by measurement of phenanthroline reactivity), while the co-presence of both cupric ions and glutathione (but not individually) spared ferrous ion levels (Fig. 5). Following complete oxidation of 20 μM ferrous ion, partial regeneration (29% in 5 min) was demonstrated by subsequent exposure to cupric sulfate (100 μM) and glutathione (400 μM), while the individual agents were either without effect or accelerated the rate of oxidation. Copper/GSH at these levels did not yield a measurable ferrous ion-like response (phenanthroline reactivity).

Copper (100 μM)/GSH (400 μM) promoted microsomal lipid peroxidation when ferric chloride was used as a source of iron (relative intensity = 9.6 ± 0.5 following a 10-min incubation, n = 4), while GSH alone was ineffective (relative intensity = 0.8 ± 0.3, n = 4).

Potential involvement of enzymes in the peroxidative response of membranes to copper/GSH/iron was explored by substituting liposomes, prepared from an extract of microsomal lipid, for microsomes in peroxidation reaction mixtures. A qualitatively similar pattern of lipid peroxidation resulted; TBA-reactivity for reaction mixtures containing the peroxidative stimuli of ferrous ion (10 μM)/GSH (400 μM) was 3.8 ± 0.9 (relative intensity; n = 4 each group) compared with the copper (100 μM)/GSH/iron peroxidative response of 6.6 ± 0.5 ($p < 0.001$) and the copper (100 μ)/GSH (400 μM) response of 0.6 ± 0.07 ($p < 0.001$).

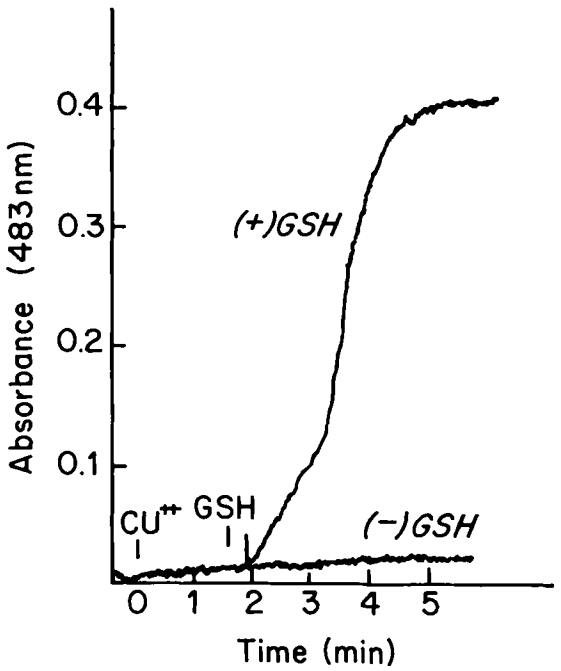

FIG. 4. GSH-mediated reduction of cupric ion. Formation of cuprous ion from cupric sulfate (100 μM) was measured spectrophotometrically at 483 nm by the formation of a complex of cuprous ion and bathocuproine (250 μM). Glutathione (400 μM) was added as indicated by the arrow. Controls (glutathione without copper) did not affect baseline absorbance.

To investigate whether copper/GSH was enhancing peroxidation by promoting propagation reactions, liposomes were prepared from fresh solutions of phosphatidylcholine (control liposomes) and from phosphatidylcholine liposomes containing 10% phosphatidylcholine hydroperoxide (oxidized liposomes). Ferrous sulfate (10 μM) promoted the formation of TBA-reactive material following incubation with oxidized liposomes, while much lower levels resulted using control liposomes. This

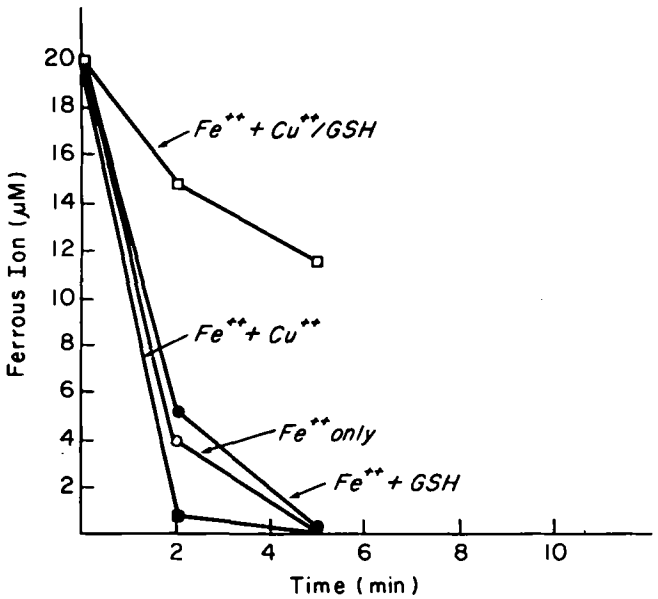

FIG. 5. Oxidation of ferrous ion during incubation at 37 C; sparing effect of copper/GSH. Ferrous sulfate (20 μM) was incubated at 37 C for the indicated duration in the presence or absence of combinations of GSH (400 μM) and cupric sulfate (100 μM) as indicated. Ferrous ion levels then were assessed by measurement of phenanthroline reactivity. Brackets denote the SEM of six samples.

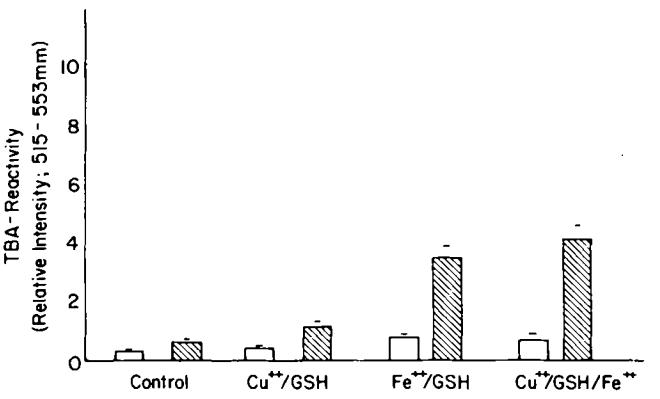

FIG. 6. Propagation of liposomal lipid peroxidation by copper and iron. Phosphatidylcholine liposomes were prepared with from egg phosphatidylcholine to which 10% phosphatidylcholine hydroperoxides were added (cross-hatched bars) or without the addition of hydroperoxides (controls, open bars). Liposomes (200 nmol/0.5 ml) were incubated in 50 mM Tris buffer (pH 7.4), with combinations of cupric sulfate (Cu^{++}, 100 μM), glutathione (GSH, 400 μM) and ferrous sulfate (Fe^{++}, 10 μM) as indicated. Lipid peroxidation was then assessed by spectrofluorometric measurement of TBA-reactivity. Brackets denote the SEM of four observations.

supports the concept that enhanced peroxidation resulted from propagation reactions (Fig. 6). By contrast, copper (50 μM)/GSH (400 μM) promoted much less peroxidation in either liposome preparation (Fig. 6).

DISCUSSION

The mechanisms of lipid peroxidation involve distinct phases of initiation (production of catalytic levels of lipid hydroperoxide accompanied by low levels of TBA-reactive end-products) and propagation (degradation of the primary lipid hydroperoxides to form reactive radicals that re-initiate peroxidation and generate additional hydroperoxide as well as end-products) (1,2). Iron functions in both the initiation and propagation phases with ADP/ferrous ion, serving primarily as an initiator and other iron chelates, including perhaps cytochrome P450-ferric ion serving as propagators. Our results suggest that in an iron-initiated system, cupric sulfate in the presence of GSH serves to augment lipid peroxidation of rat liver microsomes by a mechanism that likely involves oxidation/reduction reactions.

The potentiation of iron-induced lipid peroxidation by copper was highly dependent on the level of GSH. Cupric sulfate (100 μM) inhibited lipid peroxidation in the presence of low levels (0–200 μM) but strongly potentiated peroxidation with 400–800 μM GSH and was without effect with 2–10 mM GSH. Lower levels of copper (10 μM) effectively potentiated iron-induced peroxidation in a somewhat extended dose range (100–400 μM) of GSH. We hypothesize that GSH is required to reduce cupric ions to the cuprous form and by so doing transform copper from an inhibitor to a positive modifier of iron-induced peroxidations. Formation of cuprous ion during incubation of cupric sulfate with GSH (but not without GSH) was demonstrated by complex formation with bathocuproine supporting this contention.

Glutathione thus may promote copper-potentiated lipid peroxidation despite GSH being an inhibitor of iron-induced peroxidation (13). High level (2–10 mM) of GSH may directly reduce iron (14) and, in this manner, may promote in vitro peroxidation. Copper was ineffective as a potentiator of lipid peroxidation in the presence of these GSH levels, suggesting that cuprous ions may augment peroxidation by a similar mechanism involving iron reduction. Measurement of ferrous ion levels by the phenanthroline assay indeed revealed that ferrous ions rapidly are oxidized during model incubations and that cupric sulfate in the required presence of GSH slowed the rate of decline of ferrous ion. Regeneration of ferrous ion from media in which iron had been completely oxidized similarly was demonstrated. Further support for the role of copper/GSH in the reduction of iron was obtained by using ferric chloride as an iron source in assays of microsomal lipid peroxidation as the resulting level of TBA-reactivity was comparable with that obtained with copper/GSH/ferrous sulfate.

Recent studies support the concept that both oxidized and reduced iron are required for initiation reactions (15–17). A lag period was noted for peroxidative response to copper (in the presence of ferrous ion), which may reflect the rapid oxidation of Fe^{2+} and the subsequent regeneration of ferrous ion and formation of a ratio of Fe^{3+}/Fe^{2+} active for peroxidation. Bucher et al. (16) similarly noted lag periods for reduced iron chelates that were eliminated by the addition of chelates of oxidized iron supporting the contention that both forms of iron are required.

It is conceivable that cupric ions might function as a source of oxidized metal in addition to promoting the reduction of iron. The inhibition by cupric ions of ferrous sulfate promoted peroxidation (in the absence of GSH) would argue against such a role for copper in this system.

Inhibition of microsomal lipid peroxidation by GSH has been attributed to a glutathione-dependent microsomal protein (18) as well as numerous GSH-dependent cytosolic proteins that may be adherent to the microsomal preparation (19–21). An hypothesis that copper was promoting iron-induced peroxidation by inhibiting a component of the cytosolic defense is unlikely because liposomal membranes exhibited a similar peroxidative response.

Cuprous ions added to hydrogen peroxide (a product of phagocytic cell activities) generate reactive oxygen species including hydroxyl radicals (22). However, in biological systems copper is tightly bound to protein, including most notedly ceruloplasmin and albumin (23). Evidence has indicated that hydroxyl radicals may be generated by protein-bound copper and that such hydroxyl radicals will react rapidly at their site of generation to cause site-specific protein damage (24). It is unlikely that hydroxyl radicals are involved in the peroxidative response to copper, however, as microsomes contain protective enzymes (catalase, glutathione peroxidases) that should limit the availability of hydrogen peroxide and, in any event, in systems in which the generation of hydroxy radicals was certain, it was demonstrated that hydroxyl radicals do not participate in iron-dependent peroxidation of phospholipid liposomes (17,25). Minotti and Aust have demonstrated that the initiation of lipid peroxidation by Fe^{2+} and hydrogen peroxide is not mediated by the generated hydroxyl radicals, but is alternatively linked to H_2O_2-mediated oxidation of iron and requires both reduced and oxidized forms of iron (17).

The use of a phosphate buffer in place of Tris necessitated the chelation of copper with ADP before addition to peroxidation reaction mixtures to obtain a comparable potentiation of peroxidation. Thus, it appears that complexed rather than free copper is involved in this peroxidative response. The lack of requirement for ADP with Tris buffer may be due to an alternative Tris-copper complex, the formation of which has been suggested (26).

Our data suggests that the reduction of iron plays a major role in the potentiation of lipid peroxidation by cuprous ions. We investigated on alternative mechanisms by which cuprous ion may act as promoter of propagation reactions by studying peroxidation of phospholipid liposomes with and without phospholipid hydroperoxide. Ferrous sulfate effectively propagated the peroxidation of the oxidized liposomes, while control liposomes, which lacked sufficient hydroperoxide initiator, were peroxidized to a much lesser degree. Propagation thus could be evaluated in this system largely independent of initiation. Copper/GSH was not as effective a promoter of propagation as iron or copper/GSH/iron, indicating that the primary role of copper in the potentiation of iron-induced peroxidation is to provide enough reduced iron (and an optimal Fe^{2+}/Fe^{3+} ratio) for initiation reactions to proceed rather than to promote propagation reactions.

ACKNOWLEDGMENT

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REFERENCES

1. Svingen, B.A., O'Neal, F.O., and Aust, S.D. (1978) *Photochem. Photobiol. 28,* 803-810.
2. Svingen, B.A., Buege, J.A., O'Neal, F.O., and Aust, S.D. (1979) *J. Biol. Chem. 254,* 5892-5899.
3. Hochstein, P., Kumar, S., and Forman, S.J. (1980) *Ann. N.Y. Acad. Sci. 355,* 240-246.
4. Ding, A.H., and Chan, P.C. (1984) *Lipids 19,* 278-284.
5. Wittig, M., and Steffen, C. (1984) *Res. Commun. Chem. Pathol. Pharmacol. 44,* 477-492.
6. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-285.
7. Eskola, J., and Laakso, S. (1983) *Biochim. Biophys. Acta 751,* 305-311.
8. Hamberg, M., and Samuelsson, B. (1967) *J. Biol. Chem. 242,* 5344-5354.
9. Mihara, M., Uchiyama, M., and Fukuzawa, K. (1980) *Biochem. Med. 23,* 302-311.
10. Yagi, K. (1984) in *Methods of Enzymology* (Packer, L., ed.) Vol. 105, pp. 328-331, Academic Press, New York.
11. Kumar, K.S., Rowse, C., and Hochstein, H. (1978) *Biochem. Biophys. Res. Commun. 83,* 587-592.
12. Tien, M., Morehouse, L.A., Bucher, J.R., and Aust, S.D. (1982) *Arch. Biochem. Biophys. 218,* 450-458.
13. Pederson, T.C., Buege, J.A., and Aust, S.D. (1973) *J. Biol. Chem. 248,* 7134-7141.
14. Tien, M., Bucher, J.R., and Aust, S.D. (1982) *Biochem. Biophys. Res. Commun. 107,* 279-285.
15. Braughler, J.M., Duncan, L.A., and Chase, R.L. (1986) *J. Biol. Chem. 261,* 10282-10289.
16. Bucher, J.R., Tien, M. and Aust, S.D. (1983) *Biochem. Biophys. Res. Commun. 111,* 777-784.
17. Minotti, G., and Aust, S.D. (1986) *J. Biol. Chem. 262,* 1098-1104.
18. Hill, K.E., and Burk, R.F. (1984) *Biochem. Pharmacol. 33,* 1065-1068.
19. Ursini, F., Maiorino, M., Valente, M., Ferri, L., and Gregolin, C. (1982) *Biochim. Biophys. Acta 710,* 197-211.
20. Gibson, D.D., Hornbrook, K.R., and McCay, P.B. (1980) *Biochim. Biophys. Acta 620,* 572-582.
21. Tan, K.H., Meyer, D.J., Belin, J., and Ketterer, B. (1984) *Biochem. J. 220,* 243-252.
22. Gutteridge, J.M., and Wilkins, S. (1983) *Biochim. Biophys. Acta 759,* 38-41.
23. Gutteridge, J.M.C., Hill, C., and Blake, D.R. (1984) *Clini. Chimica. Acta 139,* 85-90.
24. Steveninck, J.V., Van der Zee, J., and Dubbelman, T.M.A.R. (1985) *Biochem. J. 232,* 309-311.
25. Gutteridge, J.M.C. (1982) *FEBS. Lett. 150,* 454-462.
26. McPhail, D.B., and Goodman, B.A. (1984) *Biochem. J. 221,* 559-560.

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Serum Lipids in Spontaneously Hypertensive Rats and Sprague-Dawley Rats Fed Menhaden Oil

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Dietary n-3 fatty acids, abundant in fish oil, exert a variety of effects that attenuate cardiovascular disease. In this study, we assessed the effect of fish oil (menhaden oil) on the serum lipid profile in hypertensive and normotensive rats. Spontaneously hypertensive rats (SHR) or Sprague-Dawley rats (SD) were fed either standard powdered diet (L-485), or L-485 + 5% menhaden oil (MO) or L-485 + 5% corn oil (CO) from weaning through eight months of age. Systolic blood pressure (BP) was periodically determined on SHR. Serum lipid profiles were performed at eight months on samples taken from the exposed hearts of anesthetized, fasted rats. SHR, compared with SD (diets combined) had significantly lower triacylglycerols (TG), higher cholesterol (CHOL), higher high density lipoprotein cholesterol (HDL CHOL), higher low density lipoprotein cholesterol (LDL CHOL), and a higher LDL:HDL ratio. Comparisons among diets (strains combined) revealed that rats fed MO had the lowest values for TG, CHOL, LDL and LDL:HDL; HDL did not vary with diet. SHR were less responsive to diet-induced changes than were SD; no decrease in TG, LDL or LDL:HDL was observed in SHR, nor was degree of hypertension altered in SHR by the MO or CO diet. In summary, MO is more effective than CO in shifting the lipid profile of rats toward one that is less atherogenic. However, the SD rat is more susceptible to diet-induced lipid modification than is the SHR.
Lipids 23, 564-567 (1988).

The relationship between the composition of dietary lipids and the development of cardiovascular disease is the subject of intense research interest. High dietary intake of saturated fatty acids and cholesterol, combined with genetic and other undefined factors, seem to contribute to the development of atherosclerosis and, perhaps, hypertension (1). Increased consumption of the polyunsaturated fatty acids found in marine fish oils (the n-3 polyenes, or linolenic acid derivatives) has been found to have a variety of beneficial effects. Early reports revealed a low incidence of cardiovascular disease among Eskimos who consume large amounts of these oils (2). The n-3 polyenes decreased platelet aggregation (3-5), and reduced levels of cholesterol and triacylglycerols in humans (6-9) and laboratory animals (10-12).

We used the spontaneously hypertensive rat (SHR) to examine the effect of chronic ingestion of marine fish oils on subjects prone to cardiovascular disease. This strain, derived from the Kyoto-Wistar (13), exhibits severe hypertension that begins to develop at four wk, and that is accompanied by vascular pathology similar to that found in humans (13-16). A moderate amount (5%) of fish oil was added to the diet of SHR and of normotensive Sprague-Dawley rats from weaning (4 wk) through eight months of age. We analyzed the effects on the lipoprotein profile of both strains, and monitored the development of hypertension in the SHR.

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Abbreviations: CHOL, cholesterol; CO, corn oil; FA, fatty acid; HDL CHOL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; MO, menhaden oil; TG, triacylglycerol; ANOVA, analysis of variance; BP, blood pressure; BW, body weight; GLM, general linear model; SAS, statistical analysis system; SBMF, South Bend Medical Foundation; SD, Sprague-Dawley rats; SHR, spontaneously hypertensive rats; SPSHR, stroke-prone spontaneously hypertensive rats.

METHODS

Experimental design. The main purpose of these experiments was to assess various effects of the addition of n-3 polyenes to the diet of rats prone to hypertension and vascular disease (SHR), and compare them with the effects of the same diet on rats that exhibit no tendency to such pathology. For comparison with SHR, we selected the Lobund Sprague-Dawley rat (SD), a strain that has been observed through 56 generations of breeding at Lobund, and has shown no tendency towards cardiovascular disease. Rats were weaned at four wk of age, at which time they were weighed and distributed into groupings that would produce a similar mean weight for each group before initiation of treatment. One group was fed a diet supplemented with menhaden oil (MO) (a marine oil rich in n-3 polyenes); a second group was fed a diet supplemented with an equivalent amount of corn oil (CO) as a control; a third group was maintained on the stock diet utilized by Lobund Laboratories (L-485) to provide an evaluation of baseline values for both strains, before the addition of oils. All rats were maintained on the diets from 4 to 35 wk. This extended period was necessary to allow adequate time for the development of cardiovascular lesions in SHR, which generally are not apparent before eight months. Systolic blood pressure (BP) and body weight (BW) were measured on SHR initially, then at two-wk intervals through 12 wk (the period of rapid increments in BP); afterwards, measurements were made at monthly intervals. All rats were killed at 35 wk of age, and blood and tissue samples were taken for evaluation.

Animals. Animals were obtained from the inbred colony at Lobund Laboratory. Original SHR stock had been obtained from the Laboratory Supply Co. (Indianapolis, IN). They were given unchlorinated tap water and housed in plastic boxes on Sani-cel bedding, in air- and humidity-controlled rooms, with a 12-hr light and dark cycle.

Diet. Before weaning, all animals were fed L-485, a natural grain diet supplemented with vitamins and minerals (Table 1) (17). After weaning, rats were maintained either on powdered L-485, or on powdered L-485 supplemented with 5%, by weight, menhaden oil (MO) (Zapata Haynie Corp., Reedville, VA) or 5% CO. A complete listing of the fatty acid composition of each diet is provided in Table 2. Rats were fed ad libitum. Food containers were emptied and replenished every 48 hr to prevent the ingestion of the toxic oxidation products of MO.

TABLE 1

Rodent Stock Diet L-485

Ingredient	Percent of diet
Ground maize	58.9625
50% Soybean oil meal	30.0
17% Alfalfa meal	3.5
Corn oil (once refined)	3.0
Salt	1.0
Dicalcium phosphate	1.0
Calcium carbonate	0.5
Lysine (feed grade)	0.5
Methionine (feed grade)	0.5
B.H.T.	0.0125
Trace mineral mix	0.025
Vitamin premix	1.0
	100.0000%
Average analysis (%)[a]	
Protein	19.92
Fat	5.28
Fiber	3.00
Moisture	11.17
Ash*	5.51
Nitrogen-free extract*	55.12

[a]Calculated on as-fed basis using pertinent ingredient composition information.

*Calculated values.

TABLE 2

Fatty Acid Composition of Diets (g/kg Diet)

	Diet		
	L-485	L-485 + MO	L-485 + CO
Fatty acid			
Saturated			
Lauric	0.05	0.04	0.04
Myristic	0.44	3.37	1.12
Palmitic	5.31	13.20	10.15
Stearic	1.45	1.68	2.88
Arachitic	0.21	0.50	0.20
Total	7.46	18.79	14.39
Monounsaturated			
Palmitoleic	0.54	8.26	1.26
Oleic	21.53	20.45	45.20
Behenic	0	0.40	0
C_{14} monoethenoic	.02	0	0
Total	22.09	29.11	46.46
Polyunsaturated			
Linoleic	21.95	20.85	38.00
Linolenic	1.46	16.22	1.42
C_{20} polyethenoic	0	0	0
C_{22} polyethenoic	0	0	0
Total	23.41	52.42	39.42
Total fatty acids	52.96	100.32	100.27

Determination of serum lipids. Triacylglycerols (TG), cholesterol (CHOL), HDL CHOL and LDL CHOL were determined in serum of rats fasted overnight. Blood samples were taken from the exposed hearts of ether-anesthetized rats and allowed to clot for 30 min before centrifugation. Some serum samples were frozen before analysis. Analysis of all samples was performed at the South Bend Medical Foundation (SBMF), South Bend, IN, using standard clinical procedures. TG were measured by a modification of the procedure of Levy and Frederickson, which is based upon depletion of NADH after a series of reactions in which TG are the initial substrate (18). CHOL measurements were done by a procedure based upon the oxidation of quinoneimine (19). Measurement of HDL CHOL was performed after precipitation of LDL and VLDL by dextran sulfate and magnesium chloride; HDL CHOL was then measured by the above method for CHOL (19). LDL CHOL was calculated according to the equation:

LDL CHOL = Total CHOL − HDL CHOL − (triacylglycerols/5).

Blood pressure determinations. Systolic blood pressure (BP) was evaluated by the tail cuff method, using the IITC Model 59 amplifier, which does not require preheating of the animal.

Statistical analysis. Data were analyzed using the Statistical Analysis System (SAS) software package (20) on the University of Notre Dame's IBM 3033 mainframe computer. A two-factor design (strain and diet) for analysis of variance (ANOVA) was performed individually on each serum lipid component, blood pressure and body weight, using the general linear model (GLM) program from SAS. Strain had two treatments: SHR and SD. Diet had three treatments: L-485, MO and CO. The GLM program compensates for unequal sample sizes. In each ANOVA procedure, a single error mean square is used to calculate a separate F-statistic for the overall model, the strain, the diet and the interaction between diet and strain treatments. Duncan's multiple-range test was used to distinguish between treatments within a factor when the GLM procedure indicated that a factor or interaction had a significant effect on the parameter being analyzed. Significance was set at 0.05. Values are expressed as Means ± SE.

RESULTS

Data from the blood lipid analyses, as well as body weight and blood pressures determined at 35 weeks of age are summarized in Table 3.

Body weight. SD rats had significantly higher body weight than SHR (459 vs 409 g), although the weight of SHR fed L-485 exceeded that of SD fed L-485 (381 g vs 354 g). With strains combined, the weight of rats fed MO was equivalent to those fed CO (452 g vs 445 g), and exceeded that of rats fed L-485 (367 g).

Blood pressure. Apparent diet-related BP differences in SHR were not significant (MO = 215.6, CO = 221.7, and L-485 = 225.8 mm Hg).

Triacylglycerols. Serum TG levels were affected by strain and diet. ANOVA showed that SHR had significantly lower serum TG than SD. Rats fed MO had significantly lower serum TG than rats fed CO or L-485. There was no interactive effect between strain and diet.

TABLE 3

Effect of Dietary Oil on SHR and Sprague-Dawley Rats*

	Body weight (g)	Systolic BP (mmHg)	Triacylglycerols (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	LDL:HDL
SD/CO (N = 6)	495.3 ± 17.1[a]	—	90.5 ± 11.6[a,b]	74.0 ± 3.7[b,c]	46.0 ± 2.8[b]	9.9 ± 3.8[b]	0.2[b,c]
SD/MO (N = 6)	509.3 ± 21.6[a]	—	74.5 ± 7.5[b]	63.2 ± 2.3[c]	42.5 ± 1.5[b,c]	5.8 ± 1.4[b]	0.1[c]
SD/L-485 (N = 5)	354.2 ± 18.5[c]	—	104.2 ± 17.5[a]	78.0 ± 2.2[b]	36.0 ± 2.0[c]	21.2 ± 2.7[a]	0.6[a]
SHR/CO (N = 10)	415.4 ± 5.4[b]	221 ± 4	41.7 ± 5.2[c]	84.2 ± 2.4[b]	48.5 ± 2.9[b]	27.3 ± 1.7[a]	0.6[a]
SHR/MO (N = 10)	417.7 ± 5.1[b]	215 ± 2	22.8 ± 3.2[c]	74.1 ± 3.1[b,c]	47.9 ± 3.1[b]	21.7 ± 2.6[a]	0.5[a]
SHR/L-485 (N = 5)	380.6 ± 13.6[b,c]	225 ± 2	26.4 ± 5.5[c]	97.4 ± 7.2[a]	65.8 ± 3.0[a]	26.4 ± 4.1[a]	0.4[a,b]

*Data from rats at age 35 wk, after maintenance on diet from age four wk.

All values are mean ± standard error; values with the differing superscript are significantly different ($p < .05$) as determined by Duncan's multiple-range test after two-factor analysis of variance.

Cholesterol. Serum CHOL levels were affected by strain and diet. ANOVA showed that SHR had significantly higher serum CHOL than SD, but the difference was small, relative to that seen for TG. SHR values were higher on each of the diets. Rats fed MO had significantly lower serum CHOL levels than rats fed CO or L-485. MO lowered CHOL in both strains, but there was no interactive effect between strain and diet.

HDL cholesterol. Serum HDL CHOL levels were affected by strain but not by diet. ANOVA showed that SHR had significantly higher HDL CHOL than SD. SHR fed L-485 had significantly higher HDL CHOL levels than all other groups.

LDL cholesterol. Serum LDL CHOL levels were affected by strain and diet. ANOVA showed that SHR had significantly higher serum LDL CHOL than SD. MO reduced serum LDL CHOL levels. There was no interactive effect between strain and diet.

LDL/HDL ratio. The ratio followed the same pattern as serum LDL. ANOVA showed that SHR had a ratio significantly higher than SD rats. MO reduced the ratio. SD rats fed MO or CO had a ratio that was significantly lower than other groups.

DISCUSSION

The data presented compare values for blood lipids in a strain of rat that exhibits hypertension (SHR), with blood lipids in an unrelated strain that is not susceptible to hypertension (SD). A survey of values obtained by others reveals considerable variation in levels of CHOL and TG for rats maintained on unmodified diets: CHOL values ranged between 28.4–79.9 mg/dl for SHR, and 40.2–83.2 mg/dl for other strains (1,21–23); TG values of 40.2 mg/dl (1) and 96.0 mg/dl (21) for SHR, and 40.0 mg/dl (23) and 63.2 mg/dl (1) for other strains have been obtained. SHR maintained on our stock diet, L-485, had somewhat higher CHOL (97.4 mg/dl) and lower TG (26.4 mg/dl); SD had CHOL of 78.0 mg/dl, but TG levels were somewhat higher than previously observed (104.2 mg/dl) (Table 3). Variations in the stock diets as well as in techniques for measurement of serum lipids could account for the wide ranges of values reported for a given strain. It should be noted that the fatty acid (FA) content of L-485 is derived entirely from vegetable sources (Table 1), and consists of approximately equal parts monounsaturated FA (oleic acid) and polyunsaturated FA (primarily linoleic acid) (Table 2). Such a diet might be expected to minimize values for CHOL and TG.

A comparison of the SHR lipid profile to that of the SD reveals that, irrespective of diet, the SHR has lower serum TG but higher CHOL, HDL CHOL, LDL CHOL, and a higher LDL:HDL ratio. Apart from the unexpectedly low level of TG, the lipid profile presented in the SHR is consistent with the atherogenesis observed in the strain, and implies that the SHR may exhibit fundamental differences in lipid metabolism that contribute to their cardiovascular pathology.

Addition of dietary fats rich in either linoleic or linolenic acid has been reported to lower both cholesterol and triacylglycerols, the linolenic acid source (fish oil) being more effective (11,24). We observed a similar trend. Enrichment of stock diet with either CO or MO lowered both TG and CHOL in SD, with MO eliciting a greater effect. A similar pattern was observed in SHR with respect to CHOL levels; however, neither diet supplement lowered TG in SHR. With respect to HDL and LDL levels, SD responded to either oil supplement with an increase in HDL and a decrease in LDL, and a consequent reduction in the HDL:LDL ratio. MO was somewhat more effective than CO in this regard. The SHR, however, was again less responsive to the effects of either oil supplement. Both oils elicited a decrease in HDL and no change in LDL, resulting in a slight increase in the LDL:HDL ratio. This ratio, generally regarded as an indicator of atherogenic potential (atherogenic index), thus remains high in SHR, following long-term addition of either linoleic or linolenic acids to their diet. Because SD, exposed to the same diet regimen, experienced a marked reduction in this index, there appear to be fundamental differences in lipid metabolism between the strains, resulting in high LDL levels in SHR despite diet modification.

Addition of MO to the diet of SHR and stroke-prone SHR (SPSHR) has been shown to attenuate the development of hypertension (25). Others have shown that supplementation of the SHR's diet with linoleic acid, a major constituent of CO, did not alter BP (26). We did not observe a BP difference between SHR fed CO vs MO (Fig. 1). At 35 weeks of age, BP was slightly, not significantly, lower in rats fed either CO or MO compared with rats fed control, L-485, diet. The stronger hypotensive

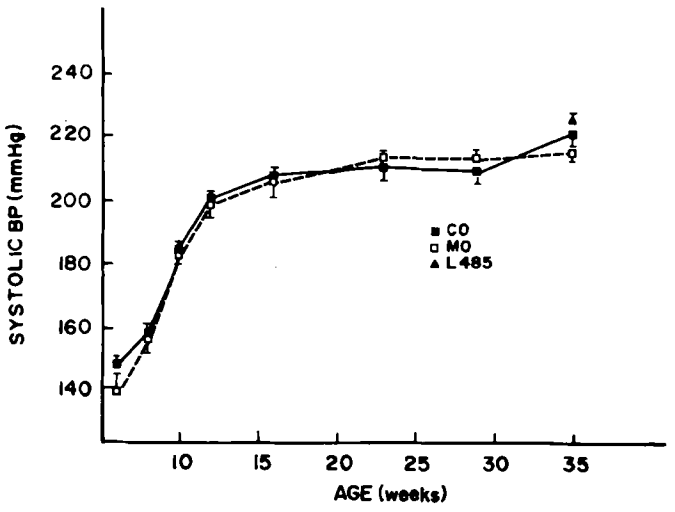

FIG. 1. Systolic blood pressure of SHR maintained on diets with differing dietary oil composition. SHR were fed either stock diet (L-485), L-485 + 5% menhaden oil (MO) or L-485 + corn oil (CO) from 4-35 wk of age. Values are mean ± S.E.

effect of MO in the previous study may be due to differences in experimental design. In that study, dietary lipid amounted to a total of 5%, either in the form of CO, or a combination of 1% CO + 4% MO. The authors speculated that this diet may have changed the ratio of n-6/n-3 fatty acids in the diet sufficiently to modify prostaglandin metabolism in a way that affected blood pressure. In our study, 5% CO or MO was added to a basic diet that already contained ca. 5% fatty acids (Table 1), primarily as soybean oil and corn oil. The superimposition of MO onto this basic diet did not result in significant BP reduction in SHR.

In summary, addition of either 5% CO or 5% MO to the diet of rats maintained on a stock diet of L-485 tends to shift the blood lipid profile towards one that is less atherogenic. MO is more effective than CO in reducing serum TG, CHOL and LDL. The SHR rat, which is genetically susceptible to hypertension and vascular pathology, is less responsive to the effects of the added lipids than the SD, exhibiting only a moderate decline in CHOL following MO, and no decline in LDL or the LDL:HDL ratio. Hypertension is not significantly ameliorated by the addition of MO to the diet. We conclude that in an animal genetically inclined toward vascular disease and hypertension, addition of MO to a diet already low in cholesterol and unsaturated fatty acids has little beneficial effect.

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REFERENCES

1. Lewis, L.A., and Naito, H.K. (1978) *Clin. Chem. 24,* 2081-2098.
2. Dyerberg, J., and Bang, H.O. (1978) *Lancet I,* 152.
3. Dyerberg, J., Bang, H.O., Stoffersen, E., Moncada, S., and Vane, J.R. (1978) *Lancet II,* 117-119.
4. Lee, T.H., Hoover, R.L., Williams, J.D., Sperling, R.I., Ravalese, J. III, Spur, B.W., Robinson, D.R., Corey, E.J., Lewis, R.A., and Austen, K.F. (1985) *N. Engl. J. Med. 312,* 1217-1223.
5. Knapp, H.R., Reilly, I.A.G., Alessandrini, P., and Fitzgerald, G.A. (1986) *N. Engl. J. Med. 314,* 937-942.
6. Phillipson, B.E., Rothrock, D.W., Connor, W.F., Harris, W.S., and Illingsworth, D.R. (1985) *N. Engl. J. Med. 312,* 1210-1216.
7. Illingsworth, D.R., Harris, W.S., and Connor, W.R. (1984) *Arteriosclerosis 4,* 270-275.
8. Harris, W.S., Connor, W.E., and McMurry, M.P. (1983) *Metabolism 32,* 179-184.
9. Mattson, F.H., and Grundy, S.M. (1985) *J. Lipid Res. 26,* 194.
10. Ruiter, A., Jongbloed, A.W., van Gent, C.H., Danse, L.H.J.C., and Metz, S.H.M. (1978) *Am. J. Clin. Nutr. 31,* 2159-2166.
11. Balasubramaniam, S., Simons, L.A., Chang, S., and Hickie, J.B. (1985) *J. Lipid Res. 26,* 684-689.
12. Gudbjarnason, S., and Oskarsdottir, S. (1977) *Biochim. Biophys. Acta 487,* 10-15.
13. Okamoto, K., and Aoki, K. (1963) *Jpn. Circ. J. 27,* 282-293.
14. Okamoto, K. (1969) *Int. Rev. Exp. Pathol. 7,* 227-270.
15. Wexler, B.C., McMurtry, J.P., and Tams, S.C. (1981) *J. Gerontol. 36,* 514-519.
16. Okamoto, K., Aoki, K., Nosaka, S., and Fukiushima, M. (1964) *Jpn. Circ. J. 28,* 943-952.
17. Kellogg, T.F., and Wostmann, B.S. (1969) *Lab. Animal Care 19,* 812-814.
18. Levy, R., and Frederickson, D.S. (1978) *Am. J. Cardiol. 22,* 576-583.
19. Warnick, R.G., Benderson, J., and Albers, J.J. (1982) *Clin. Chem. 28,* 1379-1388.
20. SAS Inst. Inc. (1985) *SAS Users Guide: Statistics,* 3rd edn., pp. 433-506, SAS Inst. Inc., Carey, NC.
21. Moon, C-K., Kang, N-Y., and Chung, J-H. (1985) *Fette Seifen Anstrichm. 87,* 74-75.
22. Yamori, Y., Nara, Y., Ikeda, K., Kihara, M., Horie, R., and Ooshima, A. (1981) *Jpn. Heart J. 22,* 465.
23. Ogawa, H., Fukushima, S., and Sasagawa, S. (1985) *Nippon Eiseigaku Zasshi 40,* 763-773.
24. Peifer, J.J., Lundberg, W.O., Ishio, W., and Warmanen, E. (1965) *Arch. Biochem. Biophys. 110,* 270-283.
25. Schoene, N.W., and Fiori, D. (1981) *Prog. Lipid Res. 20,* 569-570.
26. Singer, P., Moritz, B., Forster, D., Voigt, S., Wirth, M., Naumann, E., and Zimontkowsky, S. (1981) *Acta Biol. Med. Germ 41,* 215-225.

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Free Radicals, Antioxidants, Skin Cancer and Related Diseases

Symposium held at the 78th AOCS Annual Meeting in New Orleans, Louisiana, May 1987

Introduction to the Symposium

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The following four papers were part of a symposium on "Free Radicals, Antioxidants, Skin Cancer and Related Diseases" presented at the 78th Annual Meeting of the American Oil Chemists' Society. Although the symposium originally was designed to provide a forum for the continuing documentation of oxygen- and lipid-free radical associations with cancer and diseases of the skin, it was expanded to include some papers considering the molecular mechanisms whereby the effects observed clinically could occur. Interchange between basic and clinical researchers was expected to identify specific reactions that may cause or modify pathological processes in the complex milieu of cells and stimulate interest in using fundamental (bio)chemical research to help understand the interrelationships between free radicals, lipid oxidation, and the clinical and pathological phenomena described in papers of this symposium.

Some general background is necessary to provide the common thread connecting what may seem to be widely varying, even unrelated, research topics. Within the past decade, a considerable amount of data has been accumulated documenting the association of free radicals (particularly those derived from oxygen) and lipid oxidation with a wide variety of oxidative cytotoxicities in vivo (1) and with carcinogenesis (2,3), particularly in the promotion and progression phases of the latter (4,5). However, the precise roles of oxygen radicals and lipid oxidation in biological damage remain controversial.

That hydroxyl radicals ($OH^{\cdot}$) are very reactive and have one of the highest-known oxidation potentials is universally recognized, and much pathology is suspected of arising from this radical species that is produced in tissues primarily via metal-driven reduction of H_2O_2. However, precisely because of their high reactivity, $OH^{\cdot}$ are largely non-specific in their attack on molecules and their efficiency in terms of measurable specific damage often is low. Hence, even though production of $OH^{\cdot}$ concurrently with the development of a wide range of pathologies has been amply documented, unequivocal attribution of specific molecular or cellular changes to $OH^{\cdot}$ reactions remains difficult and, indeed, has been the subject of much controversy.

Lipid oxidation, too, has been found to occur universally in biological systems, but its precise role in specific pathologies remains unclear. Is lipid oxidation merely a result of other primary oxidations, or are alkoxyl and peroxyl-free radicals from oxidizing lipids active agents broadcasting oxidative damage? Classical biochemistry has held that oxidizing lipids contribute only indirectly to pathological processes by disrupting the packed-chain organization of membranes leading to leakiness (6,7) and/or by overwhelming cellular antioxidant defenses (8) and oxidizing the electron transport cytochromes b_5 and P-450 as well (7,9), thus allowing the unchecked progression of other oxidative reactions that normally either would not occur or would be reparable. However, in a number of pathologies lipid oxidation has been measured in cells in which no concurrent development of leakiness or depletion of antioxidant capacity occurs. Thus, the importance of these indirect mechanisms, alone, to the destructive effects of oxidizing lipids in vivo remains uncertain.

Alternatively, essential structural or functional biomolecules may be damaged directly by co-oxidation with lipids. For some years, there has been interest in the mechanisms of these reactions (10–13), and co-oxidation of critical cellular molecules, particularly proteins and nucleic acids, is considered by some researchers to be a key mechanism accounting for the biological effects of oxidizing lipids (6,14–16). Intermediate alkoxyl and peroxyl radicals of lipid oxidation react with proteins, forming protein radicals and leading to destruction of amino residues in active sites, chain scission or peptide polymerization. Some of the final breakdown products of lipid oxidation, particularly malonaldehyde, lead to co-molecular crosslinking and formation of fluorescent pigments. Enzyme destruction by oxidizing lipids also has been well-documented (6,17).

Because of the known relationship of $OH^{\cdot}$ to radiation-induced cytotoxicity and genetic damage, their potential contribution to the development of cancers is taken for granted. On the other hand, how may the reactions of oxidizing lipids relate to cancer, one of the major foci of this symposium? Lipid-mediated co-oxidation of key proteins may influence carcinogenesis epigenetically. Initiation of carcinogenesis may be affected if proteins involved in activation or detoxification of toxic agents are co-oxidized, and promotion and progression may be altered similarly when the proteins co-oxidized are those involved in replication or repair of genetic material (resulting in destabilization of gene regulation) or those controlling the replication of cells generally, thus priming cells for transformation and progression (4,18).

It now is clear that oxidizing lipids also may exert direct genetic influences on carcinogenesis as co-oxidations occur with nucleic acids. Radiomimetic free radical formation (13) and oxidative breakdown as well as lipidoxyl adduct formation in pyrimidine bases incubated with oxidizing methyl linoleate have been observed (K. M. Schaich and D. C. Borg, unpublished data), and a number of Japanese scientists have reported single-strand breaks induced in DNA by methyl linoleate hydroperoxides in model systems (19,20) and in cultured human fibroblasts (21). Strand breaks and loss of plasmid-transforming activity also were found in DNA incubated with oxidized

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microsomal lipid in liposomes (22). This activity required redox-active metals (Cu or Fe) and was inhibited by catalase, tocopherol and glutathione peroxidase but not by conventional water-soluble $OH^{\cdot}$ scavengers. Thus, lipid hydroperoxides and metal-mediated reactions in the lipid rather than aqueous phases were implicated in the DNA damage.

In considering oxidizing lipids as potential destructive agents in vivo, however, it must be remembered that lipid oxidation is a normal result of cell metabolism and tissue aging, and a variety of defenses—including glutathione peroxidase, tocopherols and membrane endocytosis—constantly are operative to control the steady-state levels of toxic hydroperoxides (7). Thus, pathological lipid oxidation requires some agents or processes capable of initiating this oxidation at levels that will provide a flux of lipid free radicals and/or other reactive oxidation products sufficient to overwhelm the rather efficient antioxidative mechanisms normally operative in vivo, and this is where free radicals from oxygen and other sources may be very important. Indeed, recognizing the critical role of initiation, papers in this symposium investigate a number of initiators that may operate physiologically, including radiation (ionizing, ultraviolet, visible light with photosensitizers); free radicals (e.g. from the metabolism of drugs and other chemicals); the "active" oxygen species $^1O_2$, H_2O_2, $O_2^{\cdot-}$, $OH^{\cdot}$; and metals. Moreover, considerable literature is accruing that strongly implicates free radicals and active oxygen species in the promotion and/or progression stages of carcinogenesis (23). This aspect of radical involvement plays an equally important role in expression of the cancerous state.

While much has been learned about the association of oxygen free radicals and of lipid oxidation with certain diseases and cancers, it is obvious that living systems are extremely complex with many reactions occurring simultaneously, and much detailed study still is needed to untangle the specific effects and relative contributions of particular damaging agents. This symposium, hopefully, contributed to a better understanding of these complicated interactions.

REFERENCES

1. Yagi, K., ed., *Lipid Peroxides in Biology and Medicine* (1982) Academic Press, New York.
2. McBrien, D.C.H., and Slater, T.F., eds., *Free Radicals, Lipid Peroxidation and Cancer* (1982) Academic Press, London.
3. Black, H.S. (1987) *Photochem. Photobiol. 46,* 213–221.
4. Troll, W., and Wiesner, R. (1985) *Ann. Rev. Pharmacol. Toxicol. 25,* 509–528.
5. Perera, M.I.R., Betschart, J.M., Virji, M.A., Katyal, S.L., and Shinozuka, H. (1987) *Toxicol. Pathol. 15,* 51–59.
6. Schaich, K.M., and Borg, D.C. (1980) in *Autoxidation in Food and Biological Systems* (Simic, M.G., and Karel, M., eds.), pp. 45–70, Plenum Press, New York.
7. Chance, B., Sies, H., and Boveris, A. (1979) *Physiol. Rev. 59,* 527–605.
8. Kappus, H., and Sies, H. (1981) *Experientia 37,* 1233–1241.
9. Bast, A., and Haenen, G.R.M.M. (1984) *Trends Biochem. Sci. 9,* 510–513.
10. Karel, M., Schaich, K.M., and Roy, R.B. (1975) *Agric. Food Chem. 23,* 159–164.
11. Schaich, K.M., and Karel, M. (1975) *J. Food Sci. 40,* 456–459.
12. Schaich, K.M., and Karel, M. (1976) *Lipids 11,* 392–400.
13. Schaich, K.M., and Borg, D.C. (1984) in *Oxygen Radicals in Chemistry and Biology* (Bors, W., Saran, M., and Tait, D., eds.), pp. 603–606, Walter de Gruyter, Berlin.
14. Borg, D.C., Schaich, K.M., Elmore, J.J. Jr., and Bell, J.A. (1978) *Photochem. Photobiol. 28,* 887–907.
15. Borg, D.C., and Schaich, K.M. (1983) in *Oxy Radicals and Their Scavenger Systems, Vol. 1: Molecular Aspects* (Cohen, G., and Greenwald, R.A., eds.), pp. 122–129, Elsevier Science, New York.
16. Borg, D.C., and Schaich, K.M. (1984) *Isr. J. Chem. 24,* 38–53.
17. Gardner, H.W. (1979) *J. Agric. Food Chem. 27,* 220–235.
18. Kensler, T.W., and Taffe, B.G. (1986) *Adv. Free Rad. Biol. Med. 2,* 347–387.
19. Inouye, S. (1984) *FEBS Lett. 172,* 231–234.
20. Ueda, K., Kobayashi, S., Morita, J., and Komano, T. (1985) *Biochim. Biophys. Acta 824,* 341–348.
21. Nakayama, T., Kaneko, M., and Kodama, M. (1986) *Agric. Biol. Chem. 50,* 261–262.
22. Akasaka, S. (1986) *Biochim. Biophys. Acta 867,* 201–208.
23. Slaga, T.J., Klein-Szanto, A.J.P., Triplett, L.L., and Yotti, L.P. (1981) *Science 213,* 1023–1025.

Fenton Reactions in Lipid Phases[1]

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Metal catalysis of membrane lipid oxidation has been thought to occur only at cell surfaces. However, conflicting observations of the pro-oxidant activity of ferric (Fe^{3+}) vs ferrous (Fe^{2+}) forms of various chelates have raised questions regarding this dogma. This paper suggests that the solubilities of iron complexes in lipid phases and the corresponding abilities to initiate lipid oxidation there, either directly or via Fenton-like production of reactive hydroxyl radicals, are critical determinants of initial catalytic effectiveness.

Partitioning of Fe^{3+} and Fe^{2+} complexes and chelates into bulk phases of purified lipids was quantified by atomic absorption spectroscopy. mM solutions of iron salts partitioned into oleic acid at levels of about micromolar. Ethylenediamine tetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) chelates were somewhat less soluble, while adenosine diphosphate (ADP) chelates, and ferrioxamine were soluble as chelates at greater than 10^{-5} M. Solubilities of all iron compounds in methyl linoleate were 10- to 100-fold lower.

To determine whether Fenton-like reactions occur in lipid phases, H_2O_2 and either Fe^{2+} or Fe^{3+} and a reducing agent were partitioned into the lipid along with the spin-trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and free radical adducts were recorded by electron paramagnetic resonance (EPR). Hydroxyl radicals ($OH^{\cdot}$) adducts were observed in oleic acid, but in lipid esters secondary peroxyl radicals predominated, and the presence of $OH^{\cdot}$ adducts was uncertain.
***Lipids 23,* 570-579 (1988).**

In the past few years, considerable attention has been given to the involvement of hydroxyl radicals ($OH^{\cdot}$), oxidizing lipids and metal ions in oxidative damage in living tissue and food systems. However, the causal relationships linking these three species to each other and to specific damage in cells have not been clarified.

Iron is a well-known catalyst of lipid oxidation, and it is also a critical reactant necessary for the non-radiolytic production of $OH^{\cdot}$ via the classical Fenton reaction, i.e. where -L represents ligand(s),

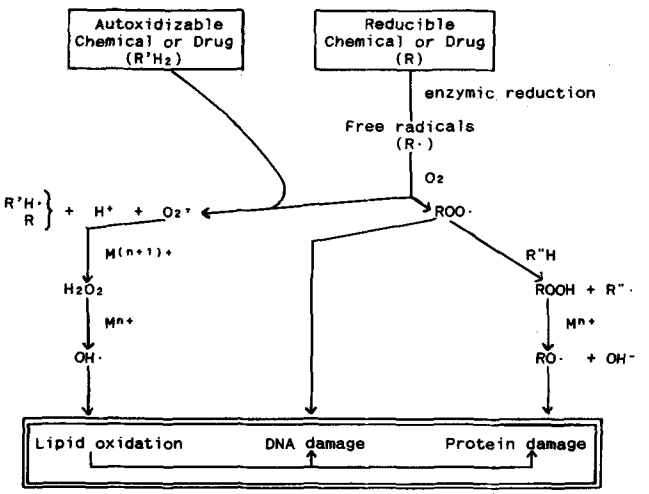
$$Fe^{2+}\text{-L} + H_2O_2 \rightarrow Fe^{3+}\text{-L} + OH^{\cdot} + OH^{-}$$

Because the non-heme redox-active iron driving this reaction is a trace constituent and there are impressive antioxidant defenses in cells, iron is most effective in initiating biological oxidations when Fe^{3+} can be recycled to Fe^{2+} by various reducing agents,

$$Fe^{3+}\text{-L} + O_2^{\cdot-}, GSH, etc. \rightarrow Fe^{2+}\text{-L} + O_2, GS^{\cdot}, etc.$$

and thus can be used catalytically to produce damaging fluxes of $OH^{\cdot}$. While it certainly is clear that the highly reactive $OH^{\cdot}$ so produced can initiate many reactions, including lipid oxidation (1-5), it is not universally accepted that the latter reaction occurs in complex biological systems.

Because many reaction sequences may occur simultaneously in cell systems or in tissues under oxidative attack (Scheme 1), the presence of oxidizing lipids may be either an effect of other free radical reactions (e.g. $OH^{\cdot}$) or it may be the cause of further molecular or cellular damage. Often the distinction is not clear. The problem of determining the causative species and the precise reaction loci of oxidative damage is further confounded by the similarity in the final expression of damage resulting from different oxygen radicals. For example, it is difficult to differentiate between effects of $OH^{\cdot}$ vs those of lipid oxyl radicals in complex biological systems. Indeed, the



SCHEME 1. Simplified scheme showing major reactive species in the pathways leading to oxidative damage to lipids, proteins and DNA in cells and tissues. Free radicals (R$^{\cdot}$) may be produced initially by either autoxidation or enzymatic reduction of suitable substrates. These radicals then may reduce O_2 to the superoxide radical anion, $O_2^{\cdot-}$, or add oxygen to form peroxyl radicals (ROO$^{\cdot}$). Metals participate as obligate reductants of H_2O_2 (usually from $O_2^{\cdot-}$ disproportionation) and organic hydroperoxides (ROOH), converting them to hydroxyl radicals ($OH^{\cdot}$) and alkoxyl radicals (RO$^{\cdot}$), respectively. These two radical species, along with ROO$^{\cdot}$, then attack critical biomolecules. When the reaction is with membrane lipids, chain reactions of lipid autoxidation ensue, and the lipid alkoxyl (LO$^{\cdot}$) and peroxyl (LOO$^{\cdot}$) radicals formed therein also can attack DNA and proteins near the site of initial $O_2^{\cdot-}$, ROO$^{\cdot}$ or RO$^{\cdot}$ reaction. The LO$^{\cdot}$ and, especially, the more stable LOO$^{\cdot}$ reaction products also can migrate some distance away to initiate additional foci of lipid chain peroxidation or to react with DNA and proteins there. Neither all intermediate steps nor all side reactions are represented, and protons and/or charges are not balanced in some reactions as written.

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Abbreviations:ADP, adenosine diphosphate; DFO, desferrioxamine; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediamine tetraacetic acid; LOOH, lipid hydroperoxides; OH, hydroxyl radicals; $O_2^{\cdot-}$, superoxide radical anion; AA, atomic absorption spectroscopy; EPR, electron paramagnetic resonance; G, gauss.

literature is replete with contradictory claims regarding the production, activity and effects of $OH^{\cdot}$ and lipid oxidation.

At least part of the difficulty in determining the interrelationships between $OH^{\cdot}$, lipid oxidation and cell damage may lie in common experimental approaches to the problem. Typically cells, sub-cellular components or liposomes are suspended in media in which Fenton components or other $OH^{\cdot}$-generating reactants are added externally or are generated in the cytosol, and then lipid oxidation or damage to protein, DNA, etc. is monitored. In most of these studies, lack of inhibition of lipid oxidation or other damage by water-soluble $OH^{\cdot}$ scavengers or metal chelators is taken as evidence that $OH^{\cdot}$ are not involved in the processes.

Similarly, in attempts to determine the mutagenicity/carcinogenicity of lipid hydroperoxides, the typical approach has utilized LOOH added to the media external to mammalian cells or Salmonella tester strains in the Ames mutagenesis assay. The failure to produce DNA strand breaks in cells or mutagenic responses in the Ames test has been interpreted as lack of toxicity or involvement in initiation or promotion of cancer by LOOH, even though in such cases the peroxides would not be expected to reach the nucleus intact, given their metabolism by cellular glutathione peroxidase or decomposition by redox-active metals (6).

We feel that in both these situations the critical site of reaction, namely, the lipid phases of membranes, has been missed. Because $OH^{\cdot}$ are extraordinarily reactive, they do not survive for more than a few collisions following their formation, and thus their reactions are intrinsically site-specific, i.e. characterized by whatever potential substrates are within 10 or a few 10s of Angstroms of where the $OH^{\cdot}$ are produced (4,7). This implies that Fenton-generated $OH^{\cdot}$ must react at the sites where iron is bound, and for effective initiation of lipid oxidation this means on the membrane surface or within the lipid environment.

The shallow penetration of a lipid bilayer that would be expected before the highly electrophilic and nonselective $OH^{\cdot}$ react provides good reason to question whether $OH^{\cdot}$ generated externally could penetrate to the hydrophobic membrane interior where the lipid chains are confined. There is evidence that iron on the membrane surface can lead to the production of some effective initiator of lipid peroxidation because iron/ascorbate reactions that are prolific sources of $OH^{\cdot}$ lead to liposome oxidation when the membrane's surface charge is neutral or negative, and iron ions are not repelled from contact or binding. This does not occur when the surface charge is positive (8). Similarly, Girotti concluded that displacement of iron from membrane-binding sites by zinc accounted for most or all of the antioxidant ability of zinc ions to protect erythrocyte ghosts from undergoing lipid peroxidation in media containing soluble Fenton systems (9).

Although there is substantial inferential evidence to support the lipid phases of membranes as the site both for Fenton-type $OH^{\cdot}$ formation in the primordial initiation of lipid oxidation and for subsequent reactions of lipid-oxyl radicals with proteins and DNA, there is little direct experimental evidence in support of that notion. Hence, we reasoned that it was important to determine experimentally whether Fenton-like formation of $OH^{\cdot}$ can occur in the lipid phase of a model system, whether $OH^{\cdot}$ so generated can initiate the free radical chains of lipid autoxidation, and if so, whether these reactions also occur in real membranes. Data demonstrating Fenton reactions in lipids phases are presented here.

MATERIALS AND METHODS

Reagents. Oleic acid was stored at room temperature, sealed, as obtained from Baker. Methyl linoleate, oleate and laurate (NuChek Prep) were stored under vacuum at −20 C. Ferrous and ferric ammonium sulphate, chloride, sulphate and oxalate were reagent grade. 1.10:1.00 (chelator/metal) chelates were prepared immediately before use by reacting ferric or ferrous ammonium sulphate with desferrioxamine (DFO) (Desferal mesylate, Ciba-Geigy), adenosine diphosphate (ADP), ethylenediamine tetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA) or sodium citrate. Hydrogen peroxide solutions (10^{-1} to 10^{-3} M) were prepared daily by dilution of a stock solution (Apache Ultrapure) that had been distilled in Vycor (gift of B.H.J. Bielski, Chemistry Dept., Brookhaven National Laboratory, Upton, NY). Concentrations were determined by the KI/molybdate/phthalate method of Allen (10). 10% (w/v) aqueous stock solutions of hydroxylamine hydrochloride (reagent grade), prepared daily, were demetalled by bathophenanthroline (11) and diluted as required. The spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Aldrich Chemical Co., Milwaukee, WI) was filtered over activated charcoal in the dark and dissolved in lipid or used directly. All solutions were prepared in water which had been purified in a Milli-Q water purification system (Millipore, minimum acceptable resistivity 12 megohms/cm), then degassed under vacuum and sparged with high-purity argon. Prepared reagents were maintained under argon until and, in most cases, during reactions and analyses. Only acid-washed glassware was used.

Partitioning of Fenton reactants into lipid phases. To effect the partitioning, equal volumes of reactants—H_2O_2, iron complexes or reducing agents (usually hydroxylamine)—were individually vortexed for two min (usually under argon) with the lipid to be studied, and the phases were separated by centrifugation.

Analysis of lipid phases. Iron partitioned into lipid phases was quantified by atomic absorption spectroscopy (Instrumentation Laboratory 551) using a graphite furnace with deuterium arc background correction. Aliquots of lipid phases were diluted 1:5 in tetrahydrofuran, and 10 μl samples were deposited in the graphite cuvette by means of a Fastac 254 autosampler. Heating and atomization sequences were programmed and controlled electronically (Instrumentation Laboratory flameless atomizer 555). Iron standards for determination of standard curves were prepared by diluting a 1000 ppm stock iron solution (12) in tetrahydrofuran and then mixing four parts of these dilutions with one part lipid (oleic acid or methyl linoleate as required) for analysis.

Fenton reactions in lipid phases. To detect radicals too short-lived or too low in concentration for direct detection by electron paramagnetic resonance (EPR), the spin-trapping technique was used, in which nitrone or nitroso compounds react with unstable radicals to give more stable nitroxide radicals. The resulting spectra frequently have characteristic features for each spin trap and can

provide information about the original radical(s) forming the adducts (2). The spin trap DMPO was dissolved directly in neat fatty acids or esters, and H_2O_2, iron compounds and hydroxylamine were separately partitioned into the same lipids in the manner described above. In most cases, both starting solutions and the lipid phases were sparged and maintained under argon to limit secondary peroxyl radical formation and background lipid oxidation. To start the reaction, aliquots from each of the lipid phases were mixed and transferred immediately to an aqueous flat cell, where subsequent radical production was monitored by EPR. During the course of these experiments, DMPO was found to be unstable when dissolved in the esters for several hours, even under argon. Thus, in subsequent studies, aliquots of neat DMPO were added directly to the Fenton reaction mixtures prepared as described above.

EPR analyses were conducted using a Varian E-12 EPR spectrometer equipped with a TE102 cavity and E-101 X-band (9.5 GHz) microwave bridge. The spectrometer is interfaced with a MassComp MC-5500 computer, which controls data taking and provides data storage and analysis as well as signal averaging of the 30-sec field scans routinely used. All scans reported in this study were 100 gauss; magnetic fields were calibrated with Fremy's salt (peroxylamine disulphonate). Hyperfine splitting constants were determined using computer simulations.

RESULTS AND DISCUSSION

In assessing the potential for Fenton reactions in lipid phases and consequential lipid oxidation, three questions must be answered: 1) Can all the reactants (hydrogen peroxide and ferrous iron or ferric iron and a reducing agent) penetrate a membrane or a lipid phase? 2) If so, does the reaction "go" in a lipid, hydrophobic environment? 3) Does a Fenton reaction in a hydrophobic environment initiate autoxidation of lipids or does any interaction with lipid remain limited to the original reaction site?

To answer these questions, model systems consisting of bulk lipids with the various reactants partitioned into them were used. Although bulk lipids are weak surrogates for structurally organized membrane bilayers with hydrophobic centers of oriented lipids, exterior polar headgroups and, in cells, external glycoprotein filaments, they may provide experimental access to environments with hydrophobicity comparable to membrane interiors. Furthermore, use of relatively simple, well-defined model systems can provide information about phase specificity and competitive kinetics that may contribute considerably to elucidating what happens when similar reactions occur in the complex milieu of cells.

Earlier work had shown differences in the proticities of bulk lipids: bulk oleic acid is weakly protic and its methyl ester and that of linoleic acid are aprotic when judged by the specific reactions of superoxide with stilbene dibromide in aprotic versus protic media (5). It was assumed that the proticity of membrane interiors might resemble that of esters, but since little data on metal solubilities in lipids was available, both bulk fatty acids and lipid esters were studied with the expectation that eventually comparisons of solubilities and/or binding of metals to different lipid classes would provide some insight regarding metal localization in membranes.

Hydroxylamine, although not very physiological, was chosen for three reasons: it is reasonably lipophilic; it was expected to be effective as a reducing agent for all the chelates to be tested (particularly ferrioxamine with a redox potential of −0.42 V vs NHE) (13 and Schaich, K.M., Linkous, C., and Borg, D.C., unpublished data); and it was expected to introduce fewer complications in the spin-trapping reactions than the more physiological reducing agents ascorbic acid, cysteine and glutathione. Ascorbic acid was unsuitable for these studies because it cannot reduce ferrioxamine (4) and it does reduce DMPO and its adducts (14). Also, as an anion it does not enter membranes or lipid phases (15). The thiols do not reduce ferrioxamine either, and they form radicals themselves and complex with oxidizing lipids in the presence of ferric iron (16).

(Question 1) Solubilities of Fenton reactants in lipid phases. Initial results using the partitioning technique indicated that mM solutions of iron salts and some low molecular weight complexes partition from water into protic oleic acid at concentrations of about μM or less (Table 1). These concentrations are 1000× or more greater than can be accounted for merely by the solubility of water in oleic acid. The recognized ability of oleic acid to complex iron may have facilitated apparent partitioning of the salts, but it is unlikely that oleic acid could compete with the strong binding of iron by the ADP chelates and ferrioxamine, which, as suggested by optical absorption spectra, partition as chelates into lipid. The resulting concentrations of ADP chelates or ferrioxamine are comparable with or greater than those of the iron salts, i.e. μM or higher for oleic acid. Iron solubilities in aprotic methyl linoleate were substantially lower, with mM solutions partitioning to final concentrations of about 10^{-7} M in the ester.

In these experiments, high concentrations of aqueous iron were used to assure that concentrations partitioned into the lipid could be detected with accuracy. Studies determining the dependence of iron solubilization in lipid on aqueous iron concentrations have not yet been completed, so it is not known whether the values reported here

TABLE 1

Partitioning of Iron into Oleic Acid and Methyl Linoleate

	Concentrations (μM) partitioned into			
	Oleic acid		Methyl linoleate	
Iron complex	Ferric	Ferrous	Ferric	Ferrous
Ammonium sulfate	5.90[a]	6.00	0.28	0.30
Sulfate	3.50	3.50	N.D.	N.D.
Chloride	3.50	3.50	N.D.	N.D.
Citrate	0.09	3.40	N.D.	N.D.
ADP[b]	>18	6.30	0.64	0.19
EDTA[b]	0.40	0.54	0.02	~0[c]
DTPA[b]	0.11	~0	~0	~0
DFO[b]	>18	N.D.	0.56	N.D.

[a]Averages of four or more determinations.

[b]Prepared with iron ammonium sulfate salts.

[c]Too low for accurate determination.

N.D., not determined.

represent true partitioning as opposed to saturation levels of iron in these lipids. Nevertheless, even 30-fold dilutions of the sub-μM iron solubilized in methyl linoleate is sufficient to fuel Fenton reactions, as will be shown later.

Contrary to what had been expected, at these aqueous concentrations the valence of the metal did not affect the solubility in lipid except for ADP chelates, where the ferric form was more lipophilic, and citrate complexes, where more iron was solubilized from the ferrous form. Because citrate binds Fe^{2+} only weakly (17), it is likely that the iron from those complexes partitioned as the ammonium sulfate salt, whereas ferric citrate is an anion (18) and thus should partition with difficulty into a hydrophobic phase. However, there presently is no obvious explanation for the behavior of the ADP chelates.

As was expected from their anionic character, EDTA and DTPA chelates have very low lipid solubility. Indeed, this low solubility combined with high binding constants for iron makes these two chelators reasonably efficient demetalling agents when the partitioning procedure is used in reverse with oleic acid and with various oils (Schaich, K. M. and Borg, D. C., unpublished data).

H_2O_2 partitioned into lipid less efficiently than did iron (Fig. 1), the partition coefficients being 1 to 2 $\times$ 10^{-3}. These coefficients are not inconsistent with those reported for H_2O_2 in chloroform (19), considering the very short equilibration period in this study. Partitioning seemed to depend on the concentration of H_2O_2 in the aqueous phase, although the apparent decreased partitioning with increasing dilution of H_2O_2 may have resulted in part from greater instability of the dilute aqueous H_2O_2 during the partitioning procedure.

Solubilization of hydroxylamine in both fatty acids and esters occurred, as was evident in spin-trapping experiments, but could not be quantified. While hydroxylamine is known to form hydroxamic acids with fatty acid esters (20), the reaction was not expected to be a complication in this experiment because it requires strongly alkaline conditions and high concentrations of hydroxylamine (21). Hydroxylamine concentrations in these reactions were limited to prevent reduction of the spin traps and the adducts. No products absorbing in the 500–560 nm range were detected.

(Question 2) Does the Fenton reaction "go" in a lipid environment? It has been shown that reactants necessary for a Fenton reaction solubilize in bulk lipids. Fenton kinetics in lipid phases now become important for assessing the potential biological significance of these reactions.

The technique of spin trapping was used to answer this question. When Fenton reactions, either with Fe^{2+} or with Fe^{3+} and a reducing agent, were run in protic oleic acid with DMPO, a four-line signal similar to that expected for the $OH^{\cdot}$ adduct was produced (Fig. 2). The splitting constants for the nitrogen (A_N) and β-hydrogen (A_H) were not equal, and thus the ratios of the line intensities were not 1:2:2:1 as seen with the $OH^{\cdot}$ adduct in water. The lower polarity of the neat lipid should decrease the splitting constants relative to those observed in water, and the splitting constants observed in oleic acid are comparable with those reported for $OH^{\cdot}$ adducts of DMPO in other organic solvents of low polarity (22), which gave six-line spectra. In this case, however, the broadening of spectral lines (i.e. increase in spectral linewidths) resulting from the high viscosity of the oleic acid is sufficient to cause overlaps in pairs of the four center lines so that an intrinsic six-line spectrum presents as a four-line first derivative spectrum with almost equal line heights, as

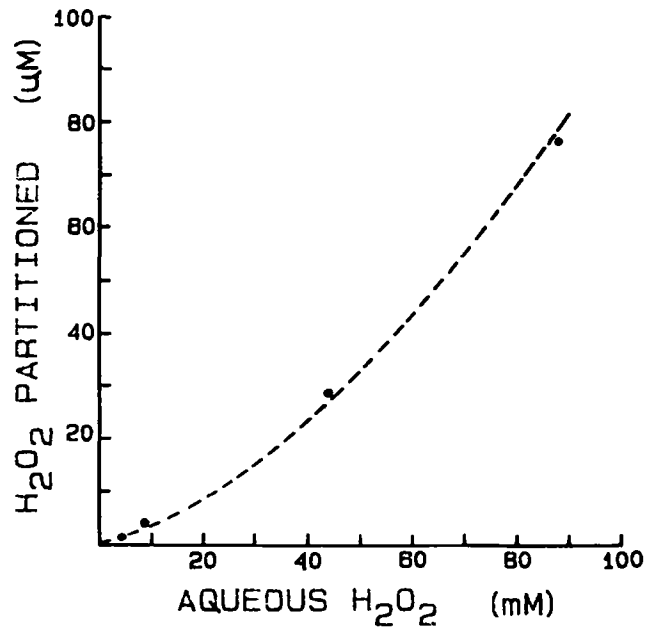

FIG. 1. Partitioning of H_2O_2 into oleic acid as a function of H_2O_2 concentrations in the aqueous phase.

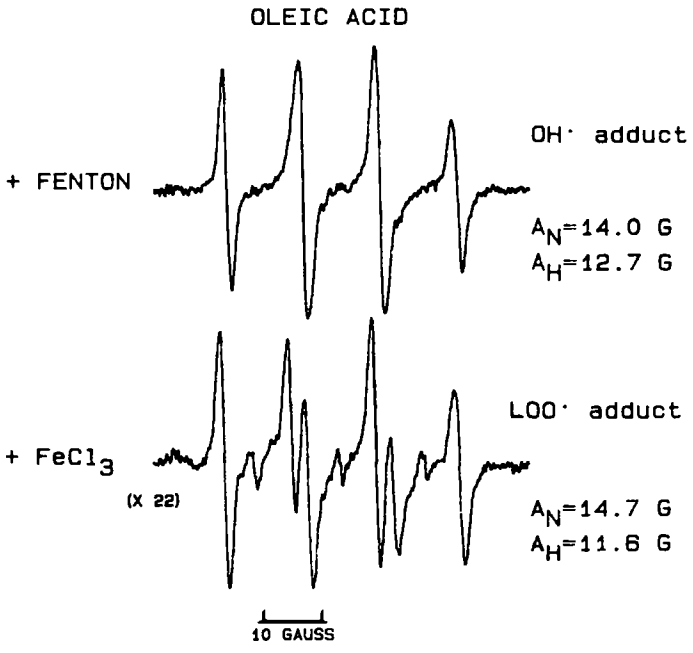

FIG. 2. Typical EPR spectra of DMPO free radical adducts in oleic acid. Top: spectrum assigned to $OH^{\cdot}$ adduct, which appeared immediately following Fenton reactions with either Fe^{2+} or Fe^{3+} plus hydroxylamine. Instrument settings: microwave power, 5 mW; modulation amplitude, 0.8 G.; gain, 3,200; time constant, 0.01 sec. Bottom: spectrum of peroxyl radical adducts obtained when $FeCl_3$ was added to oleic acid to initiate autoxidation. Instrument settings: modulation amplitude, 0.5 G.; gain, 10,000; otherwise as above.

verified by computer simulation. Thus, the assignment of this signal to $OH^{\cdot}$ adducts in oleic acid is reasonable, even though conclusive identification will require isolation and analysis of this adduct.

The signal intensities but not the line patterns were altered by moderate variations in reactant concentrations, which further indicates that primary rather than secondary radicals were being trapped under the reaction conditions of this study. Decreasing the spin-trap concentration did not increase detection of secondary lipid radicals. However, subsequent investigations have shown that when 10-fold higher iron concentrations are present, the reducing capacity of the hydroxylamine is exceeded and/or the reduction of the iron is slower than the direct reaction of ferric iron with oleic acid; in this case, iron-induced peroxyl radicals rather than $OH^{\cdot}$ are trapped.

DMPO dissolved in fresh samples of oleic acid gave no EPR signals. If oleic acid was incubated with ferric iron before DMPO was added, a lipid peroxyl radical was trapped (Fig. 2, lower trace); and if organic reducing agents such as glutathione were used or if small amounts of alcohol were present during the reaction, radicals formed by $OH^{\cdot}$ attack on them also were trapped. Ferrous iron alone in oleic acid gave no signal unless oxygen was present, in which case the $OH^{\cdot}$ adduct slowly grew in as the Fe^{2+} autoxidized and produced H_2O_2. Thus, at least qualitatively the Fenton reaction system in this lipid behaves as would be expected from classical lipid oxidation chemistry.

It is possible to follow the changes in $OH^{\cdot}$ adduct concentrations with time by fixing the magnetic field on one of the center lines of the EPR signal, and this technique was used to monitor Fenton-like reactions of ferric iron: "free" as $FeCl_3$, and chelated by ADP, EDTA and DTPA. Results are presented in Figure 3.

Of the three chelates, ADP supported Fenton kinetics in oleic acid at rates approaching that of the ferric salt, while EDTA and DTPA chelates reacted much more slowly and provided lower total adduct concentrations. There are three possible explanations for these differences: the absolute concentrations of chelates partitioned into the oleic acid were not the same, as was indicated by the AA data; EDTA and DTPA are known to react rapidly with $OH^{\cdot}$ and may have competed with DMPO for the hydroxyl radicals as they were formed close to the chelates; or the relative redox characteristics of the three chelates may be different in oleic acid than in water. Probably all of these factors contributed to the observed behavior.

That initial signal intensities for $FeCl_3$ were higher than those of the chelates reflects the greater extent of reaction that occurred within the approximately 10 sec required to transfer reaction mixtures by hand to the EPR cavity. That EDTA and DTPA chelates gave detectable $OH^{\cdot}$ production at all, despite the negligible iron concentrations detected by AA in the partitioning experiments, indicates that even sub-μM iron is sufficient to drive a Fenton reaction and/or possibly that contaminating traces of iron or other redox-active metal were present despite care taken to remove them.

Kinetics of the ferrioxamine-driven Fenton reaction in oleic acid could not be compared directly with the Fenton kinetics of those of the other chelates. Because ferrous complexes of DFO are unstable in the presence of oxygen and oxidize to ferrioxamine almost instantaneously in the presence of oxygen, the Fenton and trapping reactions with this chelate had to be performed on a vacuum manifold; samples were subsequently withdrawn from the manifold and analyzed by EPR. Hydroxylamine reduction of ferrioxamine was slow in oleic acid, but when this reaction was allowed to proceed before the addition of H_2O_2 and DMPO, a four-line adduct signal identical to that produced by the other chelates and by iron salts was observed.

When the reactions were run in aprotic lauric, oleic or linoleic methyl esters instead of the more protic acids, the expected four-line signal of hydroxyl radicals could not be detected. The signals obtained grew in more slowly than did the $OH^{\cdot}$ adducts in oleic acid, and consisted of mixtures of radical adducts (Fig. 4), the major components of which gave A_N's about 12–13.5 G and A_H's about 10–11 G. These splittings are substantially lower than those reported for $OH^{\cdot}$ adducts in organic solvents (22) but are nearly identical to those reported for lipid peroxyl radical adducts in toluene (23,24). Thus, spectral components having parameters in the range noted have been assigned to $LOO^{\cdot}$ adducts.

The $OH^{\cdot}$ adduct of DMPO in fatty acids or esters has not been reported previously, so what the spectra should look like can only be surmised from comparisons with known $OH^{\cdot}$ adducts in organic solvents of similar polarity. If the spectra of $OH^{\cdot}$ adducts in methyl linoleate and oleic acid are similar, only traces of $OH^{\cdot}$, if any, were evident in the complex spectra in methyl linoleate. If, on the other hand, the spectra of $OH^{\cdot}$ adducts in linoleate are comparable with those in ethyl acetate, benzene, or toluene (22), $OH^{\cdot}$ adducts always may be present as a

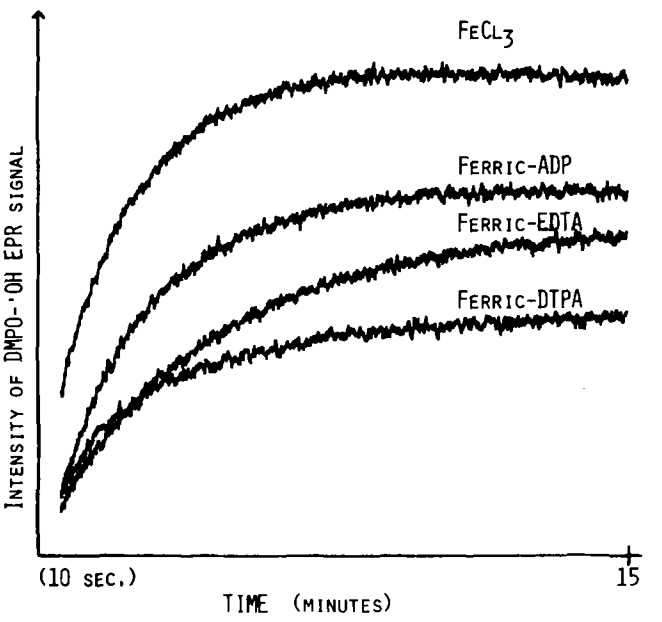

FIG. 3. Effects of iron complexation on kinetics of Fenton reactions in oleic acid: production of DMPO-$OH^{\cdot}$ adducts by Fe^{3+} "free" as its chloride salt and chelated by ADP, EDTA or DTPA. Reaction concentrations: DMPO, 44 mM; H_2O_2, 30 μM; iron compounds, as partitioned into oleic acid from 10^{-4} M aqueous solutions; plus hydroxylamine. Instrument settings: microwave power, 5 mW; modulation amplitude, 0.5 G.; gain, 8,000; time constant, 0.03 sec.

background component, contributing to the complexity and overlap of lines in the observed spectra. Indeed, computer simulations suggested that a component with $A_H \sim 12$ G and $A_H \sim 11$ G could be a component of nearly all spectra from the esters. This signal was clearest in EPR spectra from laurate and from Fe^{2+}-Fenton reactions in linoleate; in both of these situations, greater production or stability production of OH· adducts would be expected. Therefore, it is possible that this component is the OH· adduct, and attempts to isolate and identify this component conclusively are underway.

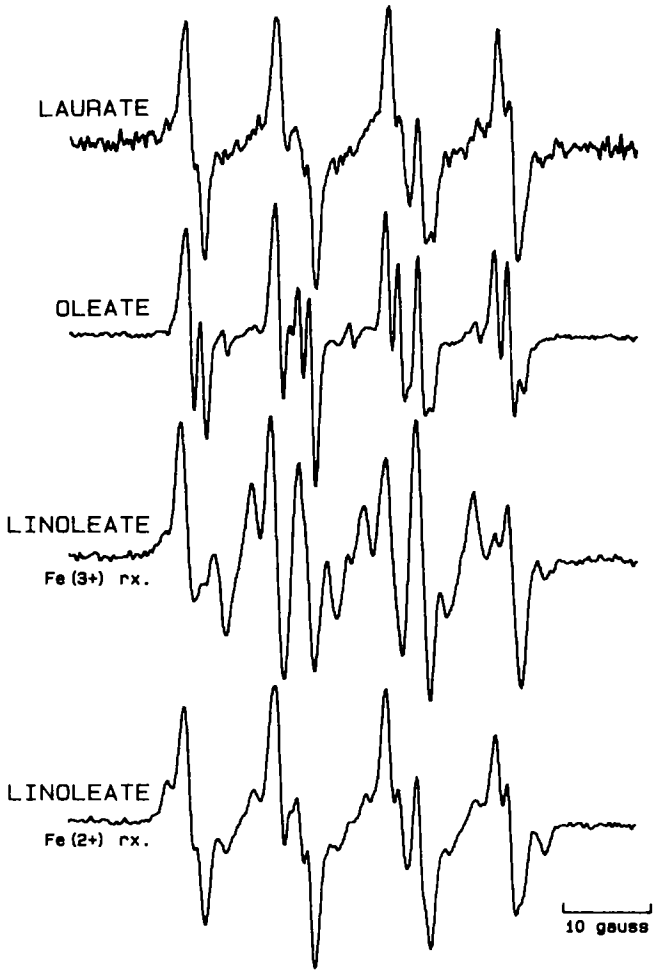

FIG. 4. Representative spectra produced during Fenton reactions in lipid esters containing hydroxylamine and 44 mM DMPO. Methyl laurate: reaction time, five min. Estimated reaction concentrations: H_2O_2, 19 μM; Fe^{3+}, 0.5×10^{-7} M. Main components: 1) $A_N = 12.82$, $^{\beta}A_H = 10.70$, A_N, $^{\gamma}A_H = 1.3$ G. (LOO· adduct); 2) $A_N = 12.1$, $^{\beta}A_H = 11.2$, $^{\gamma}A_H = 1.3$ G (unknown species). Methyl oleate: reaction time, 18 min. Estimated reaction concentrations: H_2O_2, 26 μM; Fe^{3+}, 0.1×10^{-7} M. One identified component: $A_N = 12.62$, $^{\beta}A_H = 10.25$, $^{\gamma}A_H = 1.4$ G. (LOO· adduct). Methyl linoleate (Fe^{3+}-Fenton): reaction time, 25 min. Estimated reaction concentrations: H_2O_2, 38 μM; Fe^{3+}, 0.2×10^{-7} M. Two components identified: 1) $A_N = 13.4$, $^{\beta}A_H = 10.4$ G. (unknown species); 2) $A_N = 12.82$, $^{\beta}A_H = 6.65$, $^{\gamma}A_H = 2.20$ G (LO· adduct). Methyl linoleate (Fe^{2+}-Fenton): reaction time, 7.5 min. Estimated reaction concentrations: H_2O_2, 38 μM; Fe^{2+}, 0.5×10^{-7} M. Instrument settings for all spectra: microwave power, 5 mW; modulation amplitude, 0.25 G.; gain, 8,000–10,000; time constant, 0.03 sec.

OH·-induced lipid alkyl radicals surely are being produced in these reaction systems, but it may be very difficult to trap them because these radicals react with oxygen so much faster ($k = 10^9$/M/sec) (25) than they are trapped by DMPO ($k = 10^6$/M/sec) (see review in Ref. 2). Increasing the rigor of deoxygenation with inert gases increased the radical yields and decreased oxygen line broadening, thus improving the resolution of the spectra, but it did not enhance trapping of OH· or alkyl radicals. Because of the solubility of oxygen in bulk lipids, they are extremely difficult to deoxygenate completely. Thus, trapping the initial lipid alkyl radicals may require running the reaction on a vacuum manifold to limit sufficiently the competition from oxygen or else may require utilizing a spin trap, such as nitrosobutane, which forms adducts with alkyl radicals much faster than does DMPO.

With all the esters, the DMPO adduct signals changed with time, indicating changes of radical species and varying proportions of different radicals. Methyl laurate adducts were most stable over time and required all Fenton reactants for production. As would be expected, with increasing unsaturation of the lipid, adduct mixtures became less stable and more sensitive to concentrations of Fenton reactants, particularly iron. Pulse radiolysis studies (25,26) have shown that initial OH· attack on methyl linoleate occurs indiscriminantly along the hydrocarbon chain, and that subsequent intramolecular migration of the radicals or, more likely, secondary hydrogen abstractions from other lipid molecules results in radicals and peroxides at the expected 9 and 13 carbon positions. It is likely that some of the spectral changes observed here reflect a similar process.

As in all spin-trapping experiments, caution must be exercised in interpretation of results. Both the signal intensities and adduct mixtures were quite sensitive to reactant concentrations, as shown for methyl linoleate in Figure 5. This may be expected because changing the levels of any of the Fenton reactants should alter the course of secondary branching reactions of oxidation chains, and the mix of radicals available for trapping would be altered correspondingly. In particular, high concentrations of iron (e.g. approaching μM) altered initial peroxyl radicals or their adducts so that artifactual four-line spectra similar to that of the OH· adduct were sometimes produced, either by direct attack on DMPO ($A_N = 14.98$ G, $A_H = 14.78$ G) or by conversion of peroxyl radicals or their DMPO adducts ($A_N = 14.64$ G, $A_H = 14.33$ G, see trace [E] in Figure 6). The splitting constants of these spectra are larger than would be expected for a bona fide OH· adduct in this solvent, so they are likely to arise from DMPO decomposition reactions, of which ring scission is known to produce four-line spectra resembling OH· adduct spectra (27). Similarly, when insufficient hydroxylamine was present in the reaction mixture, radicals from ferric iron-initiated lipid oxidation were trapped (c.f. trace [D], Fig. 5). While similar to the spectra obtained from full Fenton reactions, spectra from iron-dominated reactions showed altered proportions of some components and the absence of other components. Finally, a triplet ($A_N \sim 14.5$ G) was produced in systems with high iron or with long incubations; it is a nitroxide radical formed by the oxidation of DMPO.

It seems certain that Fenton processes rather than propagation reactions involving preformed lipid hydro-

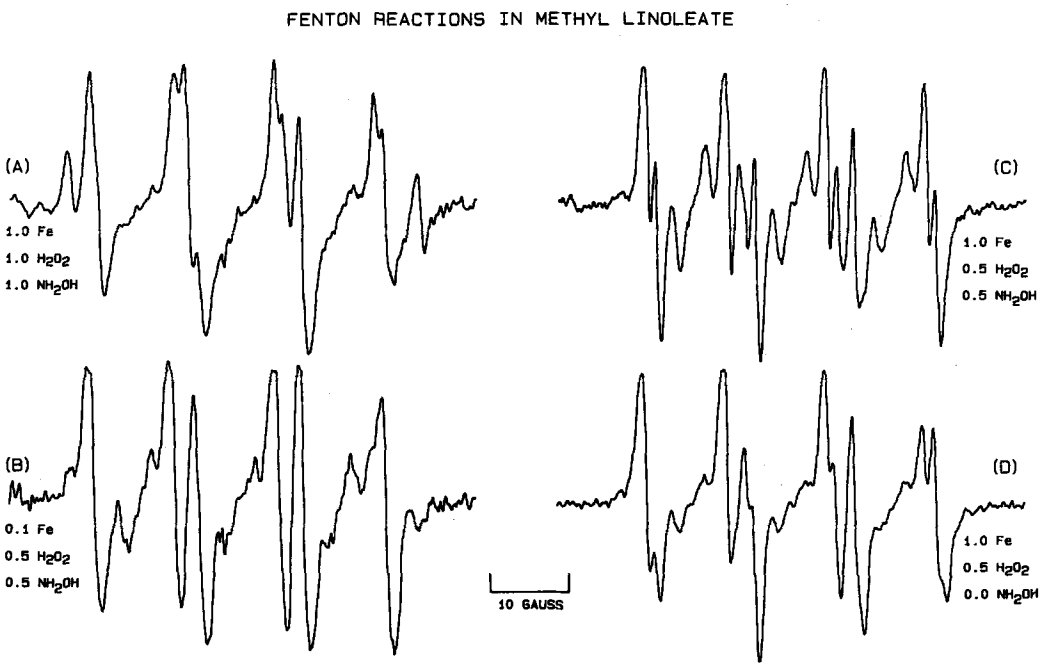

FIG. 5. Effects of varying reactant concentrations on Fenton reactions in methyl linoleate. Concentrations given are relative to (A): Fe^{3+}, 10^{-7} M; H_2O_2, 26 μM; NH_2OH, as partitioned from a 10% solution. (A) has a four-line signal with A_N = 14.98, βA_H = 14.78 G, and a component of unknown identity (X·), A_N = 13.4, βA_H = 10.4 G. The other three spectra have various mixtures of predominately X· and LOO· (A_N = 12.82, βA_H = 10.42, γA_H = 2.2 G), with lesser amounts of LO· (A_N = 12.82, βA_H = 6.65, γA_H = 2.2 G). Instrument settings comparable to those given in Figures 4 and 6.

peroxides were the dominant reactions being observed. DMPO adducts that had been identified as LO· adducts in other reaction systems either were not detected or were very weak background components when Fenton reactions were used as initiators in the esters. Peroxides were not detectable iodometrically in methyl oleate and linoleate after the partitioning step, and in incubations without Fenton reagents peroxyl adducts required substantially longer (days) to accumulate than the times typically monitored in the EPR experiments (minutes). Furthermore, variable low levels of peroxides (usually peroxide values less than 10) did not affect the spectra obtained with oleic acid, but using pre-oxidized lipid esters (e.g. peroxide values about 100 or higher) did alter the radical species trapped, particularly with methyl linoleate. DMPO added to pre-oxidized linoleate and Fenton reactions run with pre-oxidized linoleate (Fig. 6) both gave composite spectra different from those obtained in virgin (unoxidized) linoleate but similar to some spectra observed if the Fenton reactions in virgin linoleate were allowed to progress for long periods (e.g. 20 to 30 min, as in trace [C], Fig. 5). Computer simulations were used to decompose typical spectra obtained from a Fenton reaction in oxidized linoleate; this technique indicated at least three components.

One of these components had EPR hyperfine coupling constants (A_N = 12.62, βA_H = 10.27, γA_H = 1.47 G) characteristic of DMPO peroxyl adducts, although the splittings were better resolved than usual. This signal most likely results from adducts of the 9-peroxyl and/or 13-peroxyl radicals of methyl linoleate, which predominate during normal autoxidation and should not differ in their EPR spectra. The other species was present at concentrations, relative to those of the more ubiquitous peroxyl adducts, which varied with reaction time. It had a narrower beta proton splitting (A_N = 12.82, βA_H = 6.65 G) and a somewhat wider γ-proton splitting (γA_H = 2.2 G), suggestive of hyperfine spectra documented for alkoxyl radicals (2,23), and nearly identical spectra recently have been attributed to alkoxyl radicals of methyl linoleate in toluene following metalloreductions of lipoxygenase-generated hydroperoxides (24) and in freon following ozonization (28). This alkoxyl component, which should be produced only in secondary reactions of ferrous reactants with LOOH, was either absent or present only as very weak background when Fenton components were reacted in unoxidized linoleate.

The third component, a six-line spectrum with splitting constants (A_N = 13.4, A_H = 10.4 G) slightly higher than those of LOO· adducts, also was observed under other reaction conditions. Moderate to high iron concentrations, the presence of oxygen, and low-level pre-oxidation of the linoleate all seemed to increase the contribution of this species. One speculation is that this signal arises from epoxy-peroxyl radicals formed by rearrangement of alkoxyl radicals to epoxyallylic radicals that then react with O_2 (16,29). However, further work is needed to conclusively identify this radical as well as to clarify which radical species are favored under various reaction conditions.

Why could there be such differences in the Fenton reactions and/or spin-trapping of radicals in fatty acids vs their esters? Because the concentrations of iron partitioning into the esters were lower than for the acids, it is

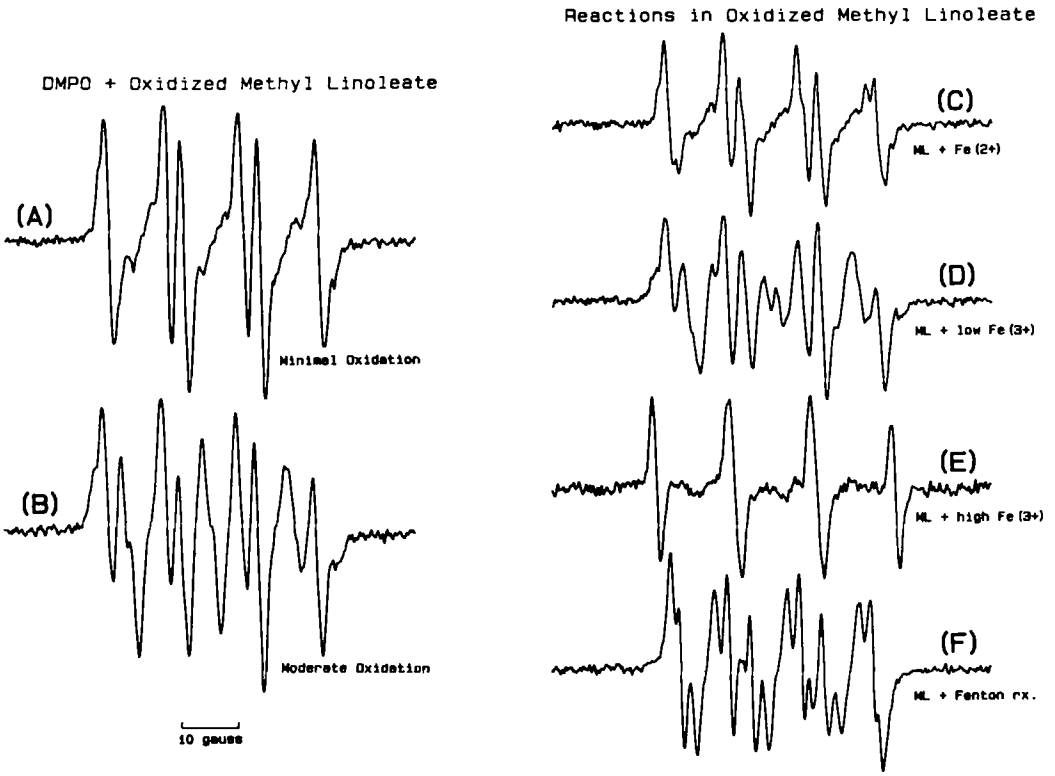

FIG. 6. Representative spectra of DMPO adducts produced by oxidizing methyl linoleate (ML) and by Fenton reactions in preoxidized ML. (A) First signal detected when ML was allowed to oxidize in the presence of DMPO (117 mM). Reaction time, approximately one day. Two identified components: 1) $A_N = 13.9$, $^{\beta}A_H = 10.9$ G (unknown species); 2) $A_N = 12.1$, $^{\beta}A_H = 11.2$, $^{\gamma}A_H = 1.3$ G (unknown species). Gain, 12,500; modulation amplitude, 0.4 G. (B) Adducts produced when DMPO was reacted with pre-oxidized ML (PV > 300). Two identified components: 1) $A_N = 13.4$, $^{\beta}A_H = 10.4$, $^{\gamma}A_H = 0.8$ G (unknown species); 2) $A_N = 14.6$, $^{\beta}A_H = 1.5$ G (unknown species). Gain, 12,500; modulation amplitude, 0.25 G. (C) Adducts from reaction of Fe^{2+} (0.05×10^{-7} M) with minimally oxidized ML (as in A). Main component: $A_N = 13.9$, $^{\beta}A_H = 10.9$ G. Gain, 10,000; modulation amplitude, 0.25 G. (D) Adducts from reaction of Fe^{3+} (0.05×10^{-7} M) with minimally oxidized ML. Reaction time six min. Three components: 1) $A_N = 13.6$, $^{\beta}A_H = 10.2$, $^{\gamma}A_H = 1.5$ G ($g = 2.0078$); 2) $A_N = 13.5$, $^{\beta}A_H = 10.1$ G ($g = 2.0068$); 3) $A_H = 14.6$, $^{\beta}A_H = 1.5$ G. All species unknown. Gain, 10,000; modulation amplitude, 0.4 G. (E) Adducts from reaction of Fe^{3+} (0.1×10^{-7} M) with ML oxidized as in (B). Reaction time, 15 min. $A_N = 14.64$, $^{\beta}A_H = 13.33$ G (from decomposition of DMPO or an adduct). Gain, 10,000; modulation amplitude, 0.25 G. (F) Adducts from Fenton reaction in minimally oxidized ML (as in A). Estimated reaction concentrations: DMPO, 215 mM; H_2O_2, 25 μM; Fe^{3+}, 0.1×10^{-7} M; plus hydroxylamine. Signal contains at least three components: 1) $A_N = 12.62$, $^{\beta}A_H = 10.27$, $^{\gamma}A_H = 1.47$ G ($LOO^{\cdot}$); 2) $A_N = 12.82$, $^{\beta}A_H = 6.65$, $^{\gamma}A_H = 2.20$ G ($LO^{\cdot}$); 3) $A_N = 13.4$, $^{\beta}A_H = 10.4$ G (unknown). Gain, 6,300; modulation amplitude, 0.1 G. Microwave power for all samples was 5 mW.

possible that Fenton reactions in the esters occurred at levels too low for efficient trapping of the primary $OH^{\cdot}$ in comparison with trapping of the secondary lipid peroxyl radicals. Alternatively, the Fenton reaction itself may proceed more slowly in aprotic media than in the protic oleic acid or in aqueous solutions. Some preliminary studies of Fenton kinetics in lipids suggest that reduction of iron is the rate-limiting step, that this step may be slower in lipid esters than in acids, and that the solubility of hydroxylamine in lipid esters may be a second critical factor affecting the overall balance of reactions in lipid esters. All of these possibilities are under investigation.

The differences in the iron solubilities and trapping patterns in oleic acid vs those in aprotic esters have some interesting implications: are there specific regions of membranes where Fenton reactions may be facilitated and are there specific preferential targets for reaction with $OH^{\cdot}$ in membranes, where most fatty acids are present as esters? Also, in pathological conditions with elevated free fatty acid levels in membranes, do the fatty acids increase iron uptake and thus predispose those membranes to potential oxidative destruction?

(Question 3) Do Fenton reactions occurring within lipid phases initiate the chain reactions of lipid autoxidation? The spin-trapping experiments suggested qualitatively that Fenton-initiated oxidation does proceed at rates considerably faster than uncatalyzed autoxidation, and subsequent experiments have provided quantification of the effects. However, the autoxidation kinetics depend in a complex manner on the form of iron complexation, both the absolute and the relative concentrations of all the

reactants (including oxygen), and the particular property measured. This work will be presented elsewhere.

Finally, in partial answer to question c posed at the close of the introduction, experiments with red cell ghosts and with liposomes have suggested that Fenton reactions occur in lipid phases of membranes (30) as well as in bulk lipids, although the form(s) and localization of the iron in the membranes have not been determined. These observations, while preliminary, nevertheless reinforce the lesson proffered by the model system studies reported here: i.e., H_2O_2, some iron complexes and DMPO all penetrate lipid phases, so membrane-localized reactions of these reagents must be expected in addition to, or perhaps even in place of, aqueous phase reactions.

SUMMARY

In summary, intramembranous formation of $OH^{\cdot}$ and initiation of lipid oxidation via Fenton reactions in the lipid phases of membranes in vivo certainly appears feasible. Clearly all the makings for compartmentalized Fenton reactions are present, and $OH^{\cdot}$ is produced in model systems using bulk lipids. Granted, these model systems do not mimic physiological conditions directly. However, the proofs-of-principle established in these chemical models should hold in vivo for cells and tissues in which H_2O_2 is being produced provided that appropriately complexed nonheme iron is available and endogenous reducing sources can replace the hydroxylamine used in the models.

Indeed, the makings for membrane-bound Fenton reactions should be available in vivo. Although little is known regarding the concentrations, endogenous ligands and cellular localization of redox-active nonheme iron, iron is known to traverse membranes by both energy-requiring and energy-independent mechanisms (31). Our solubility data certainly support the potential for the latter via passive diffusion, with accumulation of catalytically meaningful concentrations. Redox cycling of iron in membranes may be effected by thiols, by many reducing radicals (including $O_2^{\cdot-}/HO_2^{\cdot}$), and by a number of reductases (32,33). Furthermore, most of the H_2O_2 in cells, e.g. 60% or more of that produced in rat hepatocytes, is produced by membrane-bound multi-enzyme redox systems (34), and H_2O_2 produced elsewhere diffuses through membranes (35) nearly as freely as water (36).

Nevertheless, it must be emphasized that the effects resulting from Fenton reactions in membranes ultimately will be determined by the microenvironment where the redox-active metal is bound—by the character of the nearby lipid and the presence of other potential targets such as DNA or embedded proteins. Sub-μM concentrations of iron (or copper) dissolved in the hydrophobic regions of membrane lipid bilayers most likely will initiate free radical chains of lipid autoxidation, which then may proceed to damage proteins or DNA. Usually low concentrations of redox-active metal in lipid will augment lipid peroxidation in a catalytic manner. However, when the metal is bound within a few angstroms of embedded macromolecules, $OH^{\cdot}$ may attack them directly in preference to initiating a chain reaction in more distant lipid molecules of the membrane, and in such cases lipid oxidation may be decreased by the Fenton chemistry or not observed at all. Furthermore, as we recently have discussed elsewhere (37,38), it is important to keep in mind that when high concentrations of iron are used in various model systems, the metal may become a stoichiometric rather than a catalytic reactant and also may exhibit dominant chain termination behavior that is not seen at all at physiological concentrations. Observations in multiphase or compartmentalized systems will be open to misinterpretation if due consideration is not given to these possibilities.

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REFERENCES

1. Borg, D.C., Schaich, K.M., Elmore, J.J. Jr., and Bell, J.A. (1978) *Photochem. Photobiol. 28,* 887-907.
2. Schaich, K.M., and Borg, D.C. (1980) in *Autoxidation in Food and Biological Systems* (Simic, M.G., and Karel, M., eds.), pp. 45-70, Plenum Press, New York.
3. Borg, D.C., and Schaich, K.M. (1983) in *Oxy Radicals and Their Scavenger Systems. Vol. 1: Molecular Aspects* (Cohen, G., and Greenwald, R.A., eds.), pp. 122-129, Elsevier Science, New York.
4. Borg, D.C., and Schaich, K.M. (1984) *Isr. J. Chem. 24,* 38-53.
5. Borg, D.C., Schaich, K.M., and Forman, A. (1984) in *Oxygen Radicals in Chemistry and Biology* (Bors, W., Saran, M., and Tait, D., eds.), pp. 123-129, Walter deGruyter, Berlin.
6. Sies, H., and Summer, K.-H. (1975) *Eur. J. Biochem. 57,* 503-512.
7. Goldstein, S., and Czapski, G. (1986) *J. Free Rad. Biol. Med. 2,* 3-11.
8. Kunimoto, M., Inoue, K., and Nojima, S. (1981) *Biochim. Biophys. Acta 646,* 169-178.
9. Girotti, A.W., Thomas, J.P., and Jordan, J.E. (1986) *Arch. Biochem. Biophys. 251,* 639-653.
10. Allen, A.O., Hochanadel, C.J., Ghormley, J.A., and Davis, T.W. (1952) *J. Phys. Chem. 56,* 575-586.
11. Diehl, H., and Smith, G.F. (1960) *The Iron Reagents: Bathophenanthroline, 2,4,6-Tripyridyl-S-Triazine, Phenyl-2-Pyridyl Ketoxime,* G. Frederick Smith Chem. Co., Columbus, OH.
12. Willard, H.H., Merritt, L.L. Jr., and Dean, J.A. (1965) *Instrumental Methods of Analysis,* p. 353, Van Nostrand, New York.
13. Cooper, S.R., McArdle, J.V., and Raymond, K.N. (1978) *Proc. Natl. Acad. Sci. USA 75,* 3551-3554.
14. Samuni, A., Carmichael, A.J., Russo, A., Mitchell, J.B., and Riesz, P. (1986) *Proc. Natl. Acad. Sci. USA 83,* 7593-7597.
15. Kornberg, R.D., and McConnell, H.M. (1971) *Biochemistry 10,* 1111-1120.
16. Gardner, H.W., and Kleiman, R. (1981) *Biochim. Biophys. Acta 665,* 113-125.
17. Field, T.B., McCourt, J.L., and McBryde, W.A.E. (1974) *Can. J. Chem. 52,* 3119-3125.
18. Warner, R.C., and Weber, I. (1953) *J. Am. Chem. Soc. 75,* 5086-5094.
19. Walton, J.H., and Lewis, H.A. (1916) *J. Am. Chem. Soc. 16,* 633-638.
20. Keleti, G., and Lederer, W.H. (1974) *Handbook of Micromethods for the Biological Sciences,* Van Nostrand Reinhold, New York.
21. Goddu, R.F., LeBlanc, N.F., and Wright, C.M. (1955) *Anal. Chem. 27,* 1251-1255.
22. Kalyanaraman, B., Mottley, C., and Mason, R.P. (1984) *J. Biochem. Biophys. Methods 9,* 27-31.
23. Davies, M.J., and Slater, T.F. (1986) *Biochem. J. 240,* 789-795.
24. Davies, M.J., and Slater, T.F. (1987) *Biochem. J. 245,* 167-173.
25. Hasegawa, K., and Patterson, L.K. (1978) *Photochem. Photobiol. 28,* 817-823.
26. Patterson, L.K., and Hasegawa, K. (1978) *Ber. Bunsenges. Phys. Chem. 82,* 951-956.

27. Janzen, E.G. (1980) in *Free Radicals in Biology* (Pryor, W.A., ed.), Vol. 4, pp. 115–154, Academic Press, New York.
28. Pryor, W.A., Prier, D.G., and Church, D.F. (1981) *Environ. Res. 24,* 42–52.
29. Gardner, H.W., and Jursinic, P.A. (1981) *Biochim. Biophys. Acta 665,* 100–112.
30. Ding, H.A. (1984) *Copper-catalyzed Lipid Peroxidation in Erythrocyte Membranes,* PhD thesis, SUNY Downstate Medical Center, Brooklyn, NY.
31. Romslo, I., and Flatmark, T. (1973) *Biochim. Biophys. Acta 305,* 29–40.
32. Morehouse, L.A., Thomas, C.E., and Aust, S.D. (1984) *Arch. Biochem. Biophys. 232,* 366–377.
33. Winston, G.W., Feierman, D.W., and Cederbaum, A.I. (1984) *Arch. Biochem. Biophys. 232,* 378–390.
34. Ramasarma, T. (1982) *Biochim. Biophys. Acta 694,* 69–93.
35. Frimer, A., Forman, A., and Borg, D.C. (1983) *Isr. J. Chem. 23,* 442–445.
36. Chance, B., Sies, H., and Boveris, A. (1979) *Physiol. Rev. 59,* 527–605.
37. Borg, D.C., and Schaich, K.M., in *Symposium on Oxidants and Disease* (Halliwell, B., ed.), Federation of the American Societies for Experimental Biology, Bethesda, MD (in press).
38. Borg, D.C., and Schaich, K.M. in *Oxy-Radicals in MOlecular Biology and Pathology, UCLA Symposia on Molecular and Cellular Biology, New Series* (Cerutti, P.A., Fridovich, I., and McCord, J.M., eds.), Vol. 82, Alan R. Liss, Inc., New York (in press).

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Ascorbate-enhanced Lipid Peroxidation in Photooxidized Cell Membranes: Cholesterol Product Analysis as a Probe of Reaction Mechanism[1]

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Cholesterol was used as an in situ probe for studying mechanisms of lipid peroxidation in isolated erythrocyte membranes subjected to different prooxidant conditions. The membranes were labeled with [^{14}C]cholesterol by exchange with prelabeled unilamellar liposomes and photosensitized with hematoporphyrin derivative. Irradiation with a dose of blue light resulted in thiobarbituric acid-detectable lipid peroxidation that was increased markedly by subsequent dark incubation with 0.5–1.0 mM ascorbate (AH^-). Ascorbate-stimulated lipid peroxidation was inhibited by EDTA, desferrioxamine (DOX) and butylated hydroxytoluene (BHT), suggesting that the process is free radical in nature and catalyzed by membrane-bound iron. Thin layer chromatography and radiometric scanning of extracted lipids from photooxidized membranes revealed that the major oxidation product of cholesterol was the 5α-hydroperoxide (5α-OOH), a singlet oxygen adduct. Post-irradiation treatment with AH^-/Fe(III) resulted in an almost-total disappearance of 5α-OOH and the preponderance of free radical oxidation products, e.g. 7-ketocholesterol, the epimeric 7α-/7β-hydroperoxides (7α-/7β-OOH) and their respective alcohols (7α-/7β-OH). EDTA, DOX and BHT inhibited the formation of these products, while catalase and superoxide dismutase had no effect. These results are consistent with a mechanism involving 1-electron reduction of photogenerated hydroperoxides to oxyl radicals, which trigger bursts of free radical lipid peroxidation. Though generated in this system, partially reduced oxygen species, viz. superoxide, hydrogen peroxide and hydroxyl radical, appear to be relatively unimportant in the autoxidation process. *Lipids 23,* 580–586 (1988)

Cholesterol products that arise from ionizing radiation, photodynamic action and other oxidative reactions have been well-characterized in simple systems, i.e. organic solvents and artificial lipid membranes (liposomes) (1–6). With systems of this type, it has been possible to use cholesterol as a mechanistic probe for distinguishing, e.g. between singlet oxygen ($^1O_2$)-driven reactions and free radical-driven ones. We recently have advanced this area of investigation (7) by showing that cholesterol in a natural plasma membrane, the human erythrocyte ghost, can be exploited in similar fashion (8–10). Thus, two major products of oxygen radical attack, 3β-hydroxycholest-5-ene-7α-hydroperoxide (7α-OOH) and 3β-hydroxycholest-5-ene-7β-hydroperoxide (7β-OOH) generated during xanthine/xanthine oxidase treatment, were distinguished from the characteristic $^1O_2$ product, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (5α-OOH) generated by porphyrin-sensitized photooxidation. Structures of these compounds are shown in Figure 1. We now demonstrate that cholesterol can be used to detect switching from predominantly $^1O_2$-dependent lipid peroxidation to free radical-mediated peroxidation when membranes are first photooxidized in the presence of hematoporphyrin derivative (HPD) and then exposed to the electron donor ascorbate. Quantitation of these effects was made possible by tracking the products of radiolabeled membrane cholesterol.

MATERIALS AND METHODS

Materials. Freshly drawn human blood from anonymous donors was obtained from the Blood Center of Southeastern Wisconsin and used within one week. Unsealed erythrocyte membranes (white ghosts) were prepared by hypotonic lysis, followed by centrifugation and washing in lysing buffer and final resuspension in PBS (11). Total membrane protein was determined according to Lowry et al. (12) using serum albumin as the standard. During storage at 4 C, stock suspensions were blanketed with argon to minimize autoxidation. [4-^{14}C]cholesterol, 40–60 mCi/mmol in benzene, was obtained from Research Products International (Mount Prospect, IL). Hematoporphyrin derivative (HPD) was prepared according to Gomer and Dougherty (13) and partially purified by gel exclusion chromatography on Bio Gel P-10 (14). The rapidly eluting, relatively hydrophobic fraction, designated HPD-A, was used for membrane photosensitization. This fraction is enriched in tumor-localizing porphyrin aggregates that are under investigation in

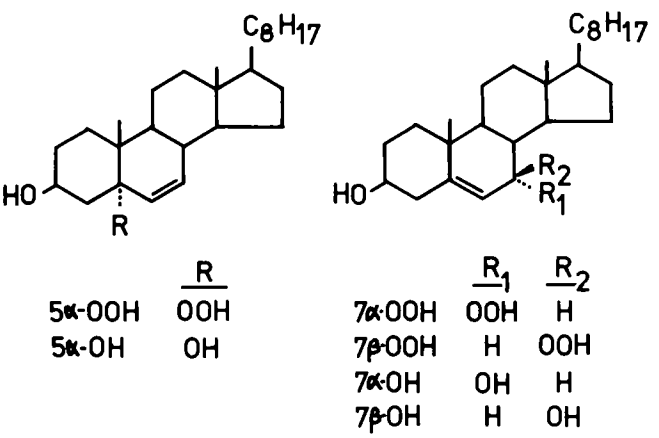

FIG. 1. Major cholesterol products derived from singlet oxygen and free radical oxidation.

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Abbreviations: AH^-, ascorbate; BHT, butylated hydroxytoluene; DOX, desferrioxamine; HPD, hematoporphyrin derivative; LOOH, lipid hydroperoxide; PBS, phosphate-buffered saline; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substance(s); TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine; 5α-OH, 5α-cholest-6-ene-3β,5-diol; 5α-OOH, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide; 7α-OH, cholest-5-en-3β,7α-diol; 7α-OOH,3β-hydroxycholest-5-en-7α-hydroperoxide; 7β-OOH, 3β-hydroxycholest-5-en-7β-hydroperoxide; 7β-OH, cholest-5-en-3β,7β-diol; 7-one, 3β-hydroxycholest-5-en-7-one.

connection with photodynamic therapy (15). HPD-A preparations in PBS were stored at −20 C until used. Porphyrin concentrations were determined spectrophotometrically, as described (14). Cholesterol, 7-ketocholesterol, egg yolk phosphatidylcholine, Cu/Zn superoxide dismutase, catalase (thymol-free), butylated hydroxytoluene, and 2-thiobarbituric acid were from Sigma Chemical Co. (St. Louis, MO), while sodium ascorbate was from BDH Chemicals (Poole, England). All other chemicals were of the highest purity available, and all aqueous solutions were prepared with deionized, glass-distilled water.

Radiolabeling of membranes. Immediately before use, [^{14}C]cholesterol plus carrier (~300 μg total; see below) was separated from any pre-existing oxidation products by thin layer chromatography (TLC) on Silica Gel-60 plates, using heptane/ethyl acetate (1:1, v/v) as the solvent system. The cholesterol zone was located by radioscanning (see below), recovered by scraping, and washed with chloroform/methanol (2:1, v/v). After centrifugation to remove gel particles, the extract was dried under argon. Cholesterol-labeled erythrocyte membranes were prepared by exchange with labeled unilamellar liposomes as follows (16). Egg phosphatidylcholine (1 μmol) and purified cholesterol (0.8 μmol, including 5-10 μCi of [^{14}C]cholesterol) were dissolved in chloroform/methanol (2:1, v/v), and the solvent was removed by flushing with argon. The cholesterol/phospholipid ratio was chosen so as to approximate that of the erythrocyte membrane (16). The lipids were suspended in PBS by vortexing for 0.5–1 min, allowed to stand for 30 min on ice, and then sonicated under a stream of argon, using a Branson Sonifier with microtip (approximately ten 30-sec doses at 30% full power, with interim chilling periods). Suspensions then were centrifuged at 27,000 × g for 10 min to remove any probe particles or large lipid aggregates. The liposomes in the supernatant fraction were mixed with ghost membranes and incubated in the presence of 2 mM azide and under argon for periods of up to 48 hr at 37 C. Ghost membrane cholesterol was typically in 15- to 20-fold excess over liposome cholesterol. Using the 48 hr-value as an end point, we found that exchange was undetectable after 3 hr and approximated 40% after 20 hr. After incubation, the ghosts were washed with 10 volumes of PBS and centrifuged at 17,000 × g for 15 min. This was repeated five to six times to ensure complete removal of liposomes and azide. The ghosts were resuspended to a final working concentration of 1 mg protein/ml (~2×10^9 ghosts/ml).

Experimental conditions. Radiolabeled ghosts were sensitized with HPD-A, transferred to a pair of thermostatted beakers (45 mm diameter) at 10 C, and irradiated from above with filtered blue light (transmittance maximum ~420 nm) (7). The suspensions were stirred continuously at a slow rate to ensure uniform light exposure. Fluence rates (W/m^2) were measured with a radiometer (Yellow Springs Instruments, Yellow Springs, OH). After a given dose of irradiation, the membranes were recovered for subsequent incubation with AH^- (without or with other additions) in the dark. These reactions were carried out at 37 C in four-place Stirrer Baths (Yellow Springs Instruments). Samples were removed for measurement of lipid peroxidation and/or TLC analysis immediately after irradiation and after one or more intervals of post-irradiation incubation.

Assessment of lipid peroxidation. Peroxidation of unsaturated lipids (excepting cholesterol and monoenoic and dienoic phospholipids) was measured by thiobarbituric acid assay, as described previously (18,19). Absorbance readings at 532 nm were converted to TBARS values for malonaldehyde and other carbonyl by-products, using an extinction coefficient of 147,000 $M^{-1}cm^{-1}$.

Chromatographic procedures. TLC separations of cholesterol photoproducts and autoxidation products were carried out in essentially the same manner as described (8). It was essential that EDTA or DOX be added to experimental samples before lipid extraction to prevent LOOH decomposition. Extracted lipids were either chromatographed directly or after borohydride reduction of hydroperoxides to their relatively stable alcohols. Stock solutions of 50 mM $NaBH_4$ in methanol/10 mM NaOH were prepared immediately before use and added in five-fold molar excess over total cholesterol. After 15 min incubation at room temperature, solvent was evaporated and the lipid residues were taken up in 10 μl of cold chloroform/methanol for application to TLC plates. Authentic 7-ketocholesterol and its borohydride reduction products, 7α-OH and 7β-OH, were used as standards for identifying free radical reaction products. Authentic 5α-OOH and its borohydride product 5α-OH were obtained by photooxidizing membranes with rose bengal, a well-known photogenerator of $^1O_2$ (20). Chromatography was performed on Silica Gel-60 F_{254} plates of 0.25-mm thickness (EM Science, Cherry Hill, NJ), using a single irrigation of heptane/ethyl acetate (1:1, v/v). Phospholipids remained at the origin in this system and therefore did not interfere with the colorimetric detection of cholesterol products (see below). Immediately after development, the plates were either sprayed with TMPD to detect peroxides, or scanned for radioactivity, using a RTLC Scanner (Model RS) equipped with a data processing system (Radiomatic Instruments, Tampa, FL). Subsequent to scanning, the plates were sprayed with 50% H_2SO_4, warmed briefly to visualize cholesterol and its various oxidation products (21) and finally photographed.

Other methods. Ascorbate was determined spectrophotometrically by measuring the reduction of Fe(III) [o-phenanthroline]$_2$ at 515 nm (22,23). Calculations were based on an extinction coefficient of 23,000 $M^{-1}cm^{-1}$ (2-electron oxidation of AH^-). Determinations were started immediately after removing samples from reaction vials. When present in assay mixtures, membranes were pelleted in a microcentrifuge, and the supernatant fluids were used for absorbance measurements.

RESULTS

Ascorbate-driven lipid peroxidation. As shown in Table 1, exposure of HPD-A-sensitized erythrocyte ghosts to a one-hr light dose resulted in a low level of TBA-detectable lipid peroxidation (~1 nmol TBARS/mg protein), which reflects free radical processes (24). Most of this reactivity probably originated from degradation of $^1O_2$-derived hydroperoxides during the assay because HPD-A has been shown to be a more efficient sensitizer of Type-II ($^1O_2$) photochemistry than Type-I (free radical) photochemistry in this system (below and [10]). Incubation of the photoperoxidized membranes with 0.5 mM AH^-/0.05 mM Fe(III) in the dark caused a strong burst of lipid

TABLE 1

Stimulation of Lipid Peroxidation in Photooxidized Membranes by Ascorbate[a]

Reaction mixture	TBARS (nmol/mg protein)			
	0 min	25 min	50 min	75 min
Control	0.0	0.0	0.4	0.3
Standard	1.0	18.7 ± 0.3	29.6 ± 0.2	28.6 ± 0.6
− Fe(III)	1.0	4.7	7.9	7.6
+ EDTA	1.0	0.9 ± 0.0	—	1.2 ± 0.1
+ DOX	1.0	0.7 ± 0.0	1.5 ± 0.1	0.8 ± 0.1
+ BHT	1.0	1.0	1.1	1.0

[a]Membranes (2 mg protein/ml in PBS) were sensitized with HPD-A (15 μg/ml) and irradiated for one hr at 10 C (fluence rate ~25 W/m²). The standard reaction mixture in subsequent dark incubations consisted of photooxidized membranes (1 mg protein/ml), AH^- (0.5 mM), and $FeCl_3$ (0.05 mM) in PBS at 37 C. In separate experiments, as indicated, the following were included: EDTA (0.06 mM), DOX (0.06 mM) or BHT (0.05 mM); or $FeCl_3$ was omitted. A control containing nonirradiated membranes, AH^- and $FeCl_3$ was incubated alongside. At the indicated times, samples were withdrawn for determination of thiobarbituric acid reactive substances (TBARS). All absorbance readings were corrected for the zero-time reading of the control. Numbers with ± designations are means ± deviation of values from duplicate experiments. —, Not determined.

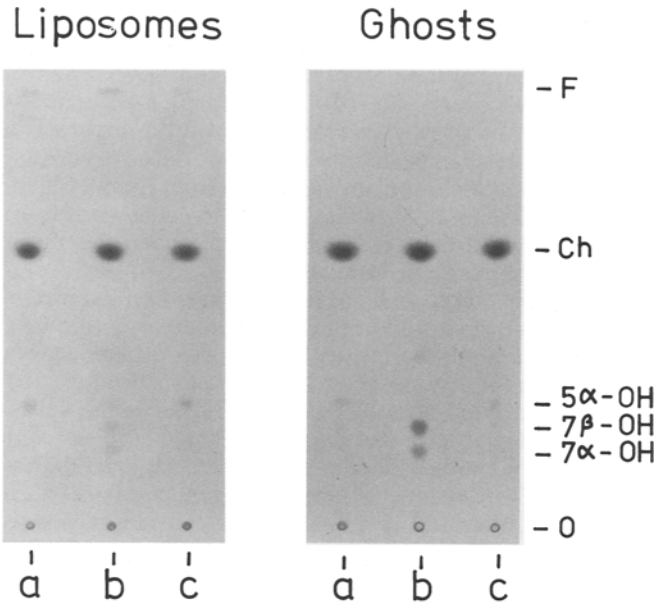

FIG. 2. Post-irradiation effects of ascorbate on cholesterol in liposomes (left) and erythrocyte ghosts (right). Preparations of [^{14}C] cholesterol-labeled liposomes and ghosts were sensitized with 5 μg/ml of HPD-A and irradiated for one hr at 10 C (fluence rate ~100 W/m²). Ghost protein was 1.0 mg/ml, corresponding to 0.7 mM phospholipid and 0.55 mM cholesterol. Liposomal lipid was approximately the same, i.e. 0.7 mM egg phosphatidylcholine and 0.5 mM cholesterol. After irradiation, the membranes were incubated for one hr at 37 C in the presence of 1 mM AH^-/10 μM $FeCl_3$ (lane b); and 1 mM AH^- /10 μ $FeCl_3$/50 μM DOX (lane c). Lane a represents irradiated membranes that were incubated one hr with $FeCl_3$, but without AH^-. Lipid extracts were reduced with $NaBH_4$ before chromatographing. Sample loads (as starting cholesterol): ~80 μg per lane. O, origin; Ch, cholesterol; F, solvent front. Spots were visualized by spraying with 50% H_2SO_4 and had the following colors: magenta (Ch); blue (5α-OH, 7α-OH, 7β-OH); gray (component between 5α-OH and Ch). Radioscans of lanes a–c (ghosts) are shown in Figure 3.

peroxidation that leveled off at approximately 30 nmol TBARS/mg protein after 40–50 min. Similar incubation of a non-photooxidized control produced no significant peroxidation, despite the fact that hydroxyl radical (OH^-), a powerful oxidant, was generated in the aqueous compartment under these conditions (23). When AH^- was omitted from the standard reaction mixture (Table 1), peroxidation remained at the starting level, indicating that Fe(III) alone could not support the reaction. The radical scavenger BHT and the chelators EDTA and DOX, which respectively stimulate and suppress iron redox cycling (25), all acted as strong inhibitors, suggesting that the reaction was free radical in nature and catalyzed by membrane-bound iron. The lower level of peroxidation observed without added iron (~8 nmol TBARS/mg at 50 min) is attributed to endogenous metal. These results are similar to earlier ones involving photooxidative priming by other sensitizers (26) and support the idea that AH^--stimulated lipid peroxidation in this system occurs via LOOH-dependent initiation (27). A similar mechanism has been proposed for reactions in which AH^- is present throughout the irradiation period (17).

Cholesterol oxidation products. Additional insights into the reaction mechanisms involved in these processes were obtained by identifying cholesterol oxidation products. TLC results obtained with [^{14}C]cholesterol-containing unilamellar liposomes and erythrocyte ghosts are shown in Figure 2. HPD-A-sensitized photooxidation (lanes a) afforded a major product that, on borohydride treatment, comigrated with the product obtained from rose bengal-sensitized photooxidation (10), and thus was identified as 5α-OOH (Fig. 1). This clearly indicates that Type-II photochemistry was dominant. In addition to 5α-OOH, small amounts of 7α-OOH and 7β-OOH must have been formed, as evidence by the presence of 7α-OH and 7β-OH. None of the diols (5α-OH, 7α-OH, or 7β-OH) were detected without borohydride treatment (see below). As indicated (8), the free radical-derived 7-OOH epimers could have arisen via a low level of Type-I photochemistry, allylic rearrangement of 5α-OOH (28), light-independent autoxidation or some combination of these processes. Some rearrangement may have occurred during post-irradiation incubation of the samples in lanes a, but this was not assessed by comparing with non-incubated samples. Also, the membrane-bound state of HPD-A (10) might have favored at least some bona fide Type-I chemistry, e.g. by electron transfer from unsaturated lipid to excited state porphyrin (8). The relative importance of these pathways has not yet been established. Incubation of photoperoxidized membranes with AH^- plus Fe(III) caused striking changes, both qualitative and quantitative, in the cholesterol product profile of liposomes as well as ghosts (Fig. 2, lanes b). For example, 5α-OH was greatly diminished (barely detectable in the ghosts) and relatively large amounts of 7α-OH/7β-OH were now evident, along with an unidentified species migrating between 5α-OH and cholesterol. The 7-diols were assigned on the basis of comigration with the reduction products of a 7-ketocholesterol standard, 7β-OH being the more abundant product (28). The appearance of 7-diols during AH^-/iron treatment clearly reflected the large burst of free radical lipid peroxidation that was observed by TBA assay (Table 1).

Formation of radical-derived products was absolutely dependent on AH^- and iron because DOX prevented the reaction (lanes c), and Fe(III) alone was ineffective. No significant amounts of 7α-/7β-OH were detected when nonirradiated membranes were exposed to AH^- and iron (not shown), which is consistent with TBARS formation being negligible under these conditions (Table 1). That a natural membrane responded similarly (albeit not identically) to an artificial bilayer in this system (Fig. 2) suggests that phospholipids per se provide the necessary sites of iron-binding and AH^- interaction, and proteins are not required. Small differences in the effect of AH^- on photooxidized liposomes and ghosts under identical reaction conditions (compare amounts of 7α-/7β-OH in lanes b, Fig. 2) could easily reflect differences in the cholesterol environment of the two membranes.

Radioscans of labeled cholesterol products from the ghost membrane experiment described in Figure 2 are shown in Figure 3. The photooxidized control (Scan A) exhibits a major peak at R_F 0.29, which coincides with the 5α-OH spot shown in Figure 2 and contains ~4% of the total radioactivity. Lesser amounts of 7α-OH (R_F 0.17) and 7β-OH (R_F 0.24) also are evident (3% and 1%, respectively). The broad peak near the origin represents an artifact of borohydride treatment that was observed as background noise in all samples, including nonirradiated samples. Treatment with AH^-/iron (Scan B) resulted in a large increase in the 7-diol peaks and decrease in the 5α-OH peak, which correspond qualitatively to the changes observed using colorimetric detection. Intensification of the peak at the origin suggests the presence of a relatively polar product that overlaps with background material (see below). An additional component (R_F 0.41; unassigned) coincides with the gray spot observed on the sprayed plate (Fig. 2). A similar product was observed in xanthine/xanthine oxidase-treated ghosts, which undergo oxyradical-dependent lipid peroxidation (8). That DOX could abolish the effects of AH^-/iron is further illustrated by Scan C, which shows a product profile that is virtually identical to that of the control (Scan A).

Radioscans taken from experiments similar to those described in Figures 2 and 3, but without borohydride treatment prior to TLC are shown in Figure 4. In this case, we were able to detect radiolabeled hydroperoxides of cholesterol as well as diols and other products (e.g. 7-one) generated during AH^--stimulated lipid peroxidation. The shift in product distribution when photoperoxidized membranes were reacted with AH^- is again strikingly evident when Scan B (before AH^-) is compared with Scan C (after AH^-/iron). The Scan B peak at R_F 0.36 (~10% of the applied radioactivity) consisted mainly of 5α-*OOH* because 5α-*OH* was the most abundant product after $NaBH_4$ treatment. In Scan C, 5α-OH (R_F 0.27) is barely evident (<1%) while 7α-OH (R_F 0.17; 4%) and 7β-OH (R_F 0.23; 2%) are relatively prominant. Additional products ascribed to free radical reactions are observed at the origin and at R_F 0.44 but are not identified. The

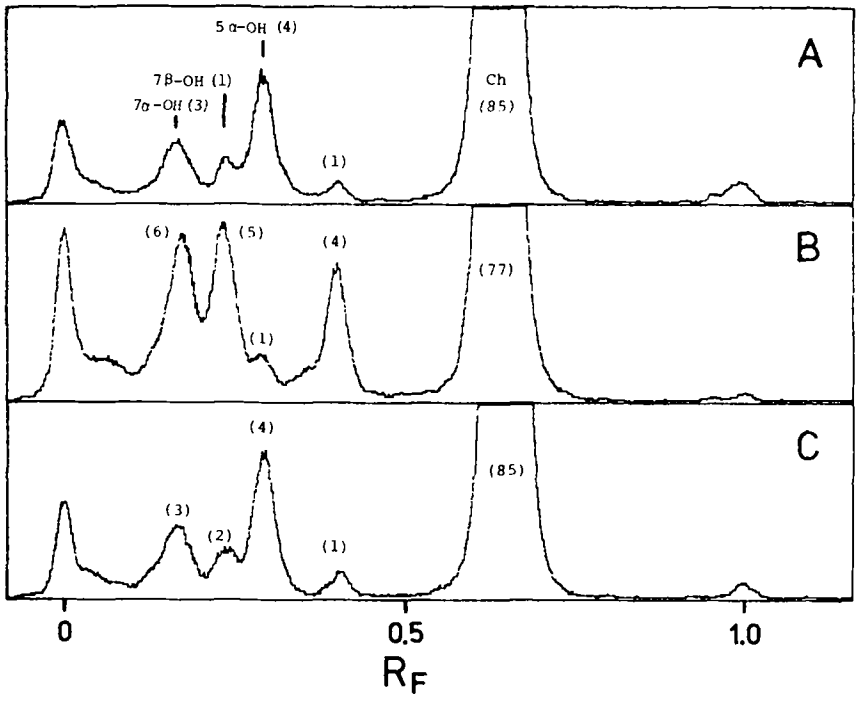

FIG. 3. Radioscans of cholesterol oxidation products from [^{14}C]cholesterol-labeled ghosts. The scans were performed on the same TLC plates as shown in Figure 2, but before H_2SO_4 spraying. (Samples were reduced with $NaBH_4$ before analyzing.) (A) photooxidized control incubated with Fe(III), without AH^-; (B) photooxidized ghosts incubated with 1 mM AH^-/10 μM Fe(III); (C) photooxidized ghosts incubated with 1 mM AH^-/10 μM Fe(III)/50 μM DOX. Sample load per lane: ~80 μg, as starting cholesterol (~2,600 cpm). Product yields (as percent total radioactivity) are shown in parentheses.

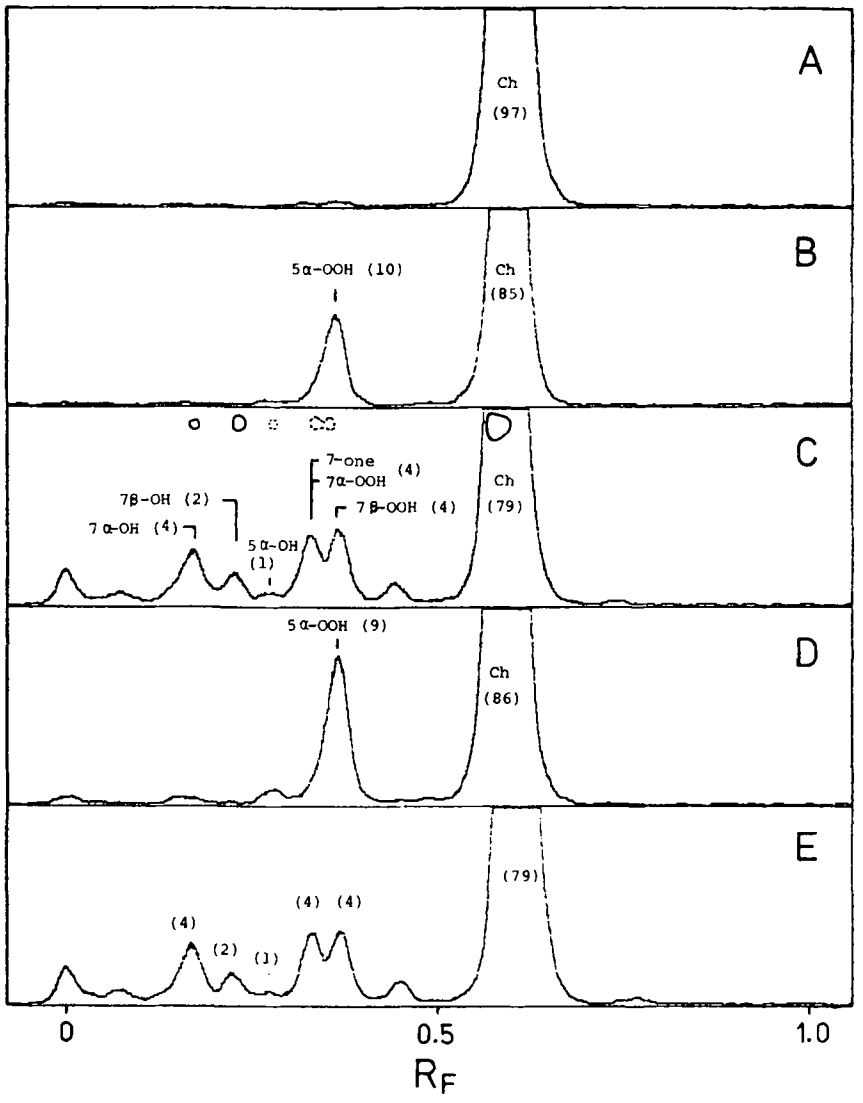

FIG. 4. Photoperoxidation of [^{14}C]cholesterol-labeled ghosts: radioscans of labeled products after exposure to ascorbate. (Samples were not reduced with borohydride before chromatographing.) The ghosts (0.5 mg protein/ml in PBS) were sensitized with HPD-A (5 μg/ml), irradiated for one hr (Fig. 2) and then dark-incubated for one hr in the presence of 1 mM AH^-/10 μM $FeCl_3$ plus the following additions: none (C); 50 μM EDTA (D); 30 μg/ml catalase (E). (A) is a 2-hr dark control; (B) is an irradiated sample before AH^- treatment. Sample load per lane: ~40 μg, as starting cholesterol (~1,200 cpm). The overlay in lane C shows spots detected with H_2SO_4. Product yields (as percent total radioactivity) are shown in parentheses.

poorly resolved doublet migrating slightly ahead of 5α-OH consists mainly of 7α-OOH and 7β-OOH (visualized by TMPD spraying and tentatively assigned as shown) plus the 7-one, which was detected by fluorescence (21). EDTA, like DOX, inhibited these reactions, as evidenced by Scan D resembling Scan B. As indicated above, this effect probably was due to chelation and removal of iron from the membrane surface, which prevented LOOH decomposition. On the other hand, catalase had no effect on the reaction (Scan E), which is consistent with the enzyme's inability to inhibit TBARS formation (17,26). Therefore, even though H_2O_2 was generated in this system and served as a precursor of $OH^\cdot$ (23), neither of these species could have been involved in the transformations described.

The cholesterol products shown in Scan B (Fig. 4) comprised part of the overall population of lipid hydroperoxides generated by photooxidation. We estimated the percentage content of cholesterol products as follows. Iodometric analysis (26) of the irradiated membranes indicated that the bulk concentration of total LOOHs (derived mainly from phospholipids and cholesterol) was 115 ± 3 μM (mean ± deviation of two determinations). Based on percentage radioactivity (corrected for 2–3% background), the yield of cholesterol hydroperoxides in Scan B was estimated to be ~38 μM. Thus, ~32 mol % of the LOOH population existed as cholesterol hydroperoxides. This figure assumes, of course, that [^{14}C]cholesterol underwent uniform exchange with membrane cholesterol and that the labeled products were representative

of the entire reactive population. Since cholesterol constitutes about 45 mol % of the erythrocyte membrane lipid (16), these data suggest that the sterol was less reactive toward photooxidation than the phospholipids. However, the difference in these values is not as great as might have been expected, considering the fact that $^1O_2$ has been shown to react with the methyl esters of linoleic, linolenic and arachidonic acids approximately 2×, 3× and 4× faster, respectively, than it reacts with cholesterol (29).

Ascorbate oxidation. Figure 5 shows AH^- decay during the incubations described in Figure 4. As can be seen, AH^- loss in the presence of membranes was accelerated considerably after photoperoxidation, confirming that LOOH-dependent oxidation of AH^- was taking place. In contrast to its effects on lipid peroxidation, EDTA stimulated AH^- oxidation, which is in agreement with previous observations (23). BHT, on the other hand, inhibited the reaction, which is consistent with the fact that this antioxidant suppressed TBA-detectable lipid peroxidation (Table 1). The protective effect of catalase is attributed to a small contribution to H_2O_2-dependent reactions to AH^- oxidation.

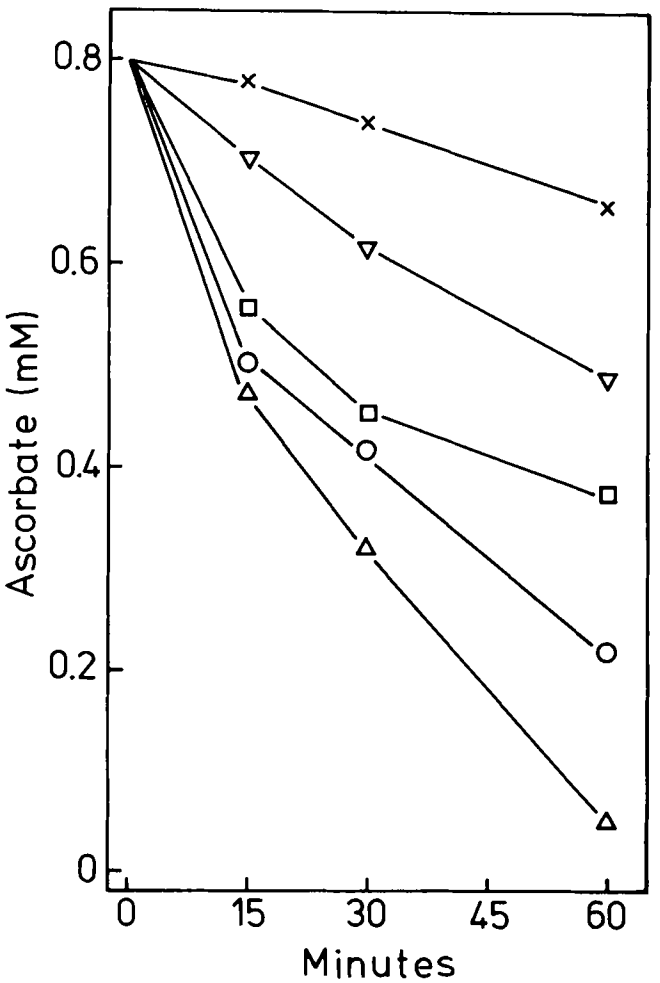

FIG. 5. Ascorbate loss during incubation with photoperoxidized membranes. Data are taken from the experiment described in Figure 4. The photooxidized ghosts were treated with AH^-/iron (○), or AH^-/iron plus the following: 50 μM EDTA (△); 30 μg/ml catalase (□); 0.2 mM BHT (▽). A control with nonirradiated membranes was run alongside (×).

DISCUSSION

The observed effects of AH^- on membranes containing preexisting LOOH is an example of hydroperoxide-dependent initiation of lipid peroxidation (27). The following reaction scheme is proposed to explain these effects (where LOOH, L·, LOO· and LO· are lipid hydroperoxide, alkyl radical, peroxyl radical and oxyl radical, respectively, and $AH^{\cdot}$. is ascorbate radical).

$$AH^- + Fe(III) \rightarrow A^{\cdot} + H^+ + Fe(II) \quad [1]$$

$$LOOH + Fe(II) \rightarrow LO\cdot + OH^- + Fe(III) \quad [2]$$

$$LH + LO\cdot \rightarrow L\cdot + LOH \quad [3]$$

$$L\cdot + O_2 \rightarrow LOO\cdot \quad [4]$$

$$LH + LOO\cdot \rightarrow L\cdot + LOOH \quad [5]$$

$$O_2 + Fe(II) \rightarrow O_2^- + Fe(III) \quad [6]$$

$$AH^- + H^+ + O_2^- \rightarrow A^{\cdot} + H_2O_2 \quad [7]$$

$$H_2O_2 + Fe(II) \rightarrow OH\cdot + OH^- + Fe(III) \quad [8]$$

Cholesterol and phospholipid hydroperoxides generated primarily by $^1O_2$ attack during porphyrin-sensitized photooxidation undergo iron-catalyzed reduction to oxyl radicals (Eqs. 1 and 2), which subsequently trigger waves of free radical lipid peroxidation (Eqs. 3–5). In this scheme, AH^- exacerbates the membrane-damaging effects of photosensitized peroxidation by reducing Fe(III) in the midst of reactive LOOH. Previous work has shown that iron-catalyzed autoxidation of AH^- in a system of this type ultimately gives rise to OH· (Eqs. 1, 6–8), which can be detected in the aqueous compartment with OH· traps (23). As indicated earlier (17,26), however, and confirmed here, OH· generated in this way (whether in the medium or on the membrane itself) has little to do with AH^--stimulated lipid peroxidation, presumably because LO· is a much more effective initiator. This derives from the fact that catalase inhibited the oxidation of OH· traps (23), but had no effect on post-irradiation lipid peroxidation (Table 1) or formation of 7α-/7β-OOH and other radical-derived products (Fig. 4).

In addition to 7α-OOH and 7β-OOH, we were able to detect 7-one by virtue of its characteristic fluorescence on ultraviolet excitation (21). As suggested in previous studies on simpler systems (28,30), the ketone may have arisen via disproportionation reactions involving two 7-peroxyl radicals or two 7-oxyl radicals (Eqs. 9 and 10, where L_2O_4 and L_2O_2 are postulated tetroxide and dioxide intermediates, and LO is the ketone). Heteroreactions involving 7-peroxyl or 7-oxyl radical and a different lipid peroxyl or oxyl radical may also have taken place.

$$2\ LOO\cdot \rightarrow [LOOOOL] \rightarrow LOH + LO + O_2 \quad [9]$$

$$2\ LO\cdot \rightarrow [LOOL] \rightarrow LOH + LO \quad [10]$$

This and the preceding study (8) represent the first real examples of how cholesterol in a natural membrane can be exploited as a reporter lipid for monitoring changes in oxidant chemistry. Numerous earlier investigations by Smith (4) and others (1–3,5,6) on cholesterol oxidation in homogeneous solution (organic solvents) and in organized media (micelles and liposomes) have provided an important background for this work. These findings improve our understanding of how AH^- and other iron reductants

might exacerbate the cytotoxic effects of photosensitizing dyes and drugs (17). In addition, they suggest new approaches for enhancing the therapeutic potency of HPD and other antineoplastic photosensitizers (15).

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REFERENCES

1. Schenck, G.O. (1957) *Angew. Chem. 69,* 579-599.
2. Suwa, K., Kimura, T., and Schaap, A.P. (1977) *Biochem. Biophys. Res. Commun. 75,* 785-792.
3. Nakano, M., Sugioka, K., Nakamura, T., and Oki, T. (1980) *Biochim. Biophys. Acta 619,* 274-286.
4. Smith, L.L. (1981) *Cholesterol Autoxidation,* Plenum Press, New York.
5. Foote, C.S., Shook, F.C., and Abakerli, R.B. (1984) *Methods Enzymol. 105,* 36-47.
6. Sevanian, A., and McLeod, L.L. (1987) *Lipids 22,* 627-636.
7. Lamola, A.A., Yamane, T., and Trozzolo, A.M. (1973) *Science 179,* 1131-1133.
8. Girotti, A.W., Bachowski, G.J., and Jordan, J.E. (1987) *Lipids 22,* 401-408.
9. Kalyanaraman, B., Feix, J.B., Sieber, F., Thomas, J.P., and Girotti, A.W. (1987) *Proc. Natl. Acad. Sci. USA 84,* 2999-3003.
10. Thomas, J.P., Hall, R.D., and Girotti, A.W. (1987) *Cancer Lett. 35,* 295-302.
11. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) *Biochemistry 10,* 2606-2617.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
13. Gomer, C.J., and Dougherty, T.J. (1979) *Cancer Res. 39,* 146-151.
14. Kessel, D., and Chow, T. (1983) *Cancer Res. 43,* 1994-1999.
15. Dougherty, T.J. (1987) *Photochem. Photobiol. 45,* 879-889.
16. Lange, Y., Cutler, H.B., and Steck, T.L. (1980) *J. Biol. Chem. 255,* 9331-9337.
17. Girotti, A.W., Thomas, J.P., and Jordan, J.E. (1985) *Photochem. Photobiol. 41,* 267-276.
18. Girotti, A.W., and Thomas, J.P. (1984) *J. Biol. Chem. 259,* 1744-1752.
19. Girotti, A.W., Thomas, J.P., and Jordan, J.E. (1986) *Arch. Biochem. Biophys. 251,* 639-653.
20. Foote, C.S. (1976) in *Free Radicals in Biology* (Pryor, W.A., ed.) Vol. 2, pp. 85-134, Academic Press, New York.
21. Smith, L.L., Mathews, W.S., Price, J.C., Bachman, R.C., and Reynolds, B. (1967) *J. Chromatogr. 27,* 187-205.
22. Shinar, E., Navok, T., and Chevion, M. (1983) *J. Biol. Chem. 258,* 14778-14783.
23. Bachowski, G.J., and Girotti, A.W., *Free Rad. Biol. Med., in press.*
24. *Pryor, W.A., Stanley, J.P., and Blair, E. (1986) Lipids 11,* 370-379.
25. Halliwell, B., and Gutteridge, J.M.C. (1984) *Methods Enzymol. 105,* 49-56.
26. Girotti, A.W., Thomas, J.P., and Jordan, J.E. (1985) *Arch. Biochem. Biophys. 236,* 238-251.
27. Aust, S.D., and Svingen, B.A. (1982) in *Free Radicals in Biology* (Pryor, W.A., ed.) Vol. 5, pp. 1-28, Academic Press, New York.
28. Teng, J.I., Kulig, M.J., and Smith, L.L. (1973) *J. Chromatogr. 75,* 108-113.
29. Doleiden, F.H., Farenholtz, S.R., Lamola, A.A., and Trozzolo, A.M. (1974) *Photochem. Photobiol. 20,* 519-521.
30. Made Gowda, N.M., and Smith, L.L. (1984) *J. Steroid Biochem. 20,* 917-922.

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Sunlight, Melanogenesis and Radicals in the Skin[1]

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Melanocytes are cells of neural crest origin residing at the dermal-epidermal juncture. They produce specialized organelles called melanosomes within which the biochemical processes of melanization occurs. UV radiation is capable of inducing melanogenesis and, during the biosynthesis of melanins, several of the putative precursors "leak out" of the melanosome and can be detected in the skin, serum and urine of individuals undergoing active melanogenesis. Most notable are the cysteinyldopas (formed by nucleophilic addition of cysteine to dopaquinone) and several dihydroxyindoles (formed by intramolecular cyclization of dopaquinone). These catechols often are methylated in the melanocyte to afford a mixture of the monomethoxy derivatives and, in some cases, the dimethoxy species.

Recent investigations in our laboratories have demonstrated that the cysteinyldopas, dihydroxyindoles, and their various methylated derivatives are photochemically unstable. Irradiation with biologically relevant ultraviolet radiation (i.e. wavelengths >300 nm) results in the rapid destruction of the precursors/metabolites and the production of a variety of free radical species. The photochemistry and potential photobiological significance of melanogenic intermediates is discussed.
Lipids 23, 587-591 (1988).

Melanin is an ubiquitous biopolymer of quinone and hydroquinone monomer units found throughout the animal and plant kingdoms. In animals, it contains concentrations of metal ions and free radicals under most known conditions and is largely responsible for pigmentation. In fact, melanins are the only pigments apart from hemoglobin in the blood that are synthesized and used extensively by mammals. The amount, type and distribution of melanin in keratinocytes determines hair and skin pigmentation and is largely responsible for basic photoprotection.

Specialized dendritic cells of neural crest origin known as melanocytes produce and package human epidermal melanin pigments. These cells reside at the dermal/epidermal junction and together with the surrounding keratinocytes form a unit that is called the epidermal melanin unit. Within these units, the average ratio of melanocytes to keratinocytes is 1:36, but it varies in different parts of the body. Melanin formation occurs in melanosomes, which are subcellular organelles transferred to keratinocytes via the dendritic processes during pigmentation. The size of the individual melanosomes dictates their distribution within keratinocytes. Melanosomes smaller than 0.8 μm are found in groups of two or more within the keratinocytes in membrane-limited vesicles called "melanosome complexes" in Caucasoids and Mongoloids, while melanosomes larger than 0.8 μm are packaged singly within the keratinocytes in membrane-limited vesicles in Negroids. Once the keratinocytes are full of melanosomes, they are pushed towards the surface of the skin where they die and ultimately are sloughed off.

Proteins sequestered from the smooth and the rough endoplasmic reticula, the Golgi apparatus and the Golgi-associated endoplasmic reticulum of melanocytes are deposited into initially unpigmented membrane-limited vesicles called premelanosomes. As the melanosome translocates to the tips of the dendrites, pigment formation slowly occurs and is nearly complete when transfer of the melanosome to the keratinocyte takes place (see Fig. 1). Delayed tanning is stimulated by the UVB component of sunlight and encompasses melanosome formation, pigment production and melanosome transfer. The detailed photobiological mechanisms of sunlight induced melanogenesis are not clearly understood.

Melanins and photoprotection. Regardless of melanin's original evolutionary function, there is little doubt that it photoprotects the skin of present-day humans (2). In their 1974 paper, "The role of natural photoprotective agents in human skin," Pathak and Fitzpatrick concluded, "Melanin and the distribution of melanosomes in the epidermis are the single most important factors in the protection of human skin from the effects of ultraviolet light." Other investigators have substantiated this view over the past 30 years (3-8). Pathak and Fitzpatrick also proposed a variety of mechanisms to explain how melanins accomplish this photoprotection, including the filtering and attenuation of impinging radiation by scattering, absorption and subsequent dissipation (as heat),

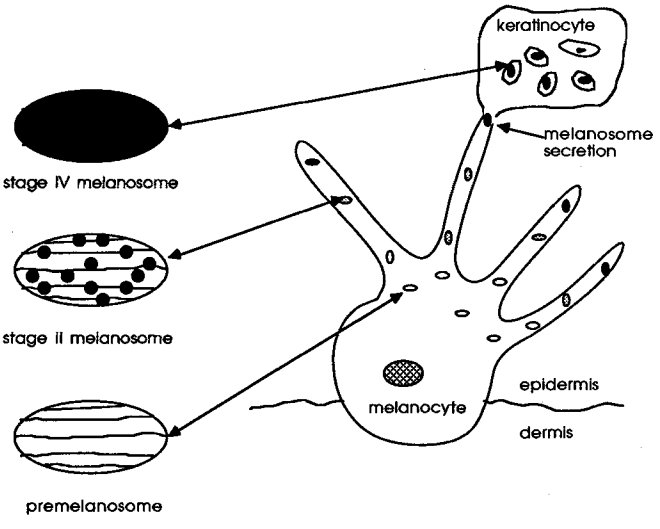

FIG. 1. Schematic representation of melanosome migration and melanization within the melanocyte and transfer to a keratinocyte.

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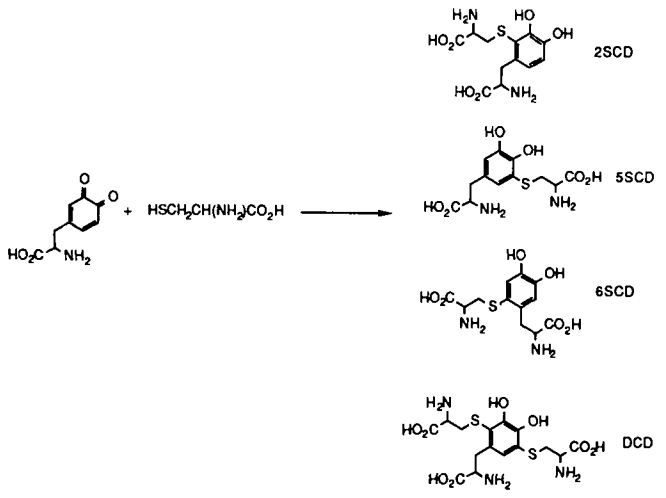
Abbreviations: COMT, catechol-O-methyl transferase; DCD, 2,5-S,S'-dicysteinyldopa; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DMICA, 5,6-dimethoxyindole-2-carboxylic acid; DMPO, 5,5-dimethyl-1-pyrolline *N*-oxide; 5(or 6)-HMICA, 5(or 6)-monomethoxyindole-2-carboxylic acid; PUVA, 8-methoxypsoralen plus UVA; 5SCS, 5-S-cysteinyldopa; UVA, (B, C) ultraviolet light in the A (B or C) region, 400-320 nm (320-280 nm or <280 nm); ESR, electron spin resonance.

absorption accompanied by redox reactions and absorption accompanied by electron transfer processes.

When considering normal, physiological and pathological effects of sunlight on human skin, differences in genetic background must be considered. For example, Negroes are much less susceptible to general actinic damage and UV-induced skin cancer than dark-complexioned Caucasians who are less susceptible than fair-skinned Caucasians. The morphology of Caucasian vs Negro epidermal melanosomes may cause much of the difference between the respective melanins. In Caucasian skin, the "melanosome complexes" are taken up by lysosomes and decomposed during passage of the host keratinocyte to the surface (9), unlike the larger, single, melanosomes of the Negro. Thus, while the horny layer in Negro skin contains intact melanosomes that effectively protect the underlying layers of living cells, this layer in Caucasians possesses little more than a dust of melanin.

Melanin metabolites. Raper was the first to postulate that indole derivatives are formed as intermediates in melanization (10). These derivatives arise from the oxidation of the 1,4-addition product of tyrosinase-generated dopaquinone (10,11). Derived forms of the two types of dihydroxyindoles formed during eumelanogenesis, i.e. 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), have been experimentally implicated to form the heteropolymer eumelanin, along with dopa, dopaquinone or both (12,13).

Prota and Nicolaus conducted in vitro studies of pheomelanin biochemistry, which demonstrated that the precursors of pheomelanin are nucleophilic addition products of cysteine and tyrosinase-generated dopaquinone (14, 15). 5-S-cysteinyldopa (5SCD) (16), the major product of this reaction, was chemically synthesized and characterized to enable great strides to be taken in pheomelanin chemistry and melanocyte biochemistry. Two-S- and 6-S-cysteinyldopa, natural 5 SCD isomers and the di-addition product, 2,5-S,S′-dicysteinyldopa (DCD) (see Scheme 1) also are formed in melanosomes (17,18). The two chemically distinct biopolymers, eu- and pheomelanin, appear to occur as an intimate mixture in the human epidermis (19).



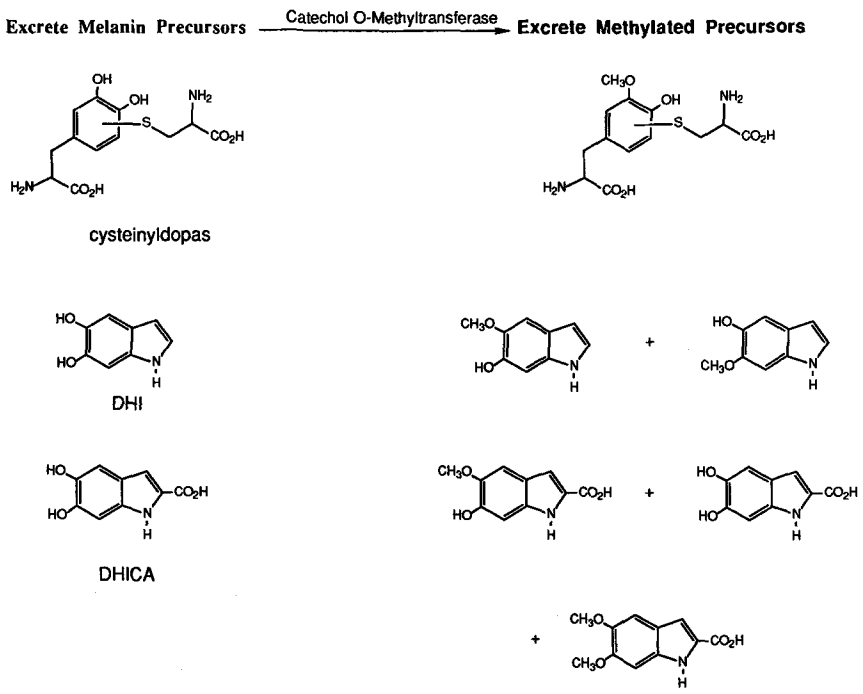
SCHEME 1. Formation of cysteinyldopas from dopaquinone and cysteine.

Detection of free dihydroxyindoles and cysteinyldopas in the urine and serum of normal humans and melanoma patients indicated their escape from the melanocyte during polymerization (20). DHI and DHICA may be O-methylated by the action of catechol-O-methyl transferase (COMPT) in the melanocyte, like many catechols, to respectively yield the 6-monomethoxyindole-2-carboxylic acid (6HMICA), 5-monomethoxyindole-2-carboxylic acid (5HMICA) and 5,6-dimethoxyindole-2-carboxylic acid (DMICA) positional isomers (Scheme 2) (21–25). Similarly, cysteinyldopas catabolized by O-methylation and/or glucuronide and sulfate conjugation (26) appear to represent detoxification products that are not cytotoxic.

The role of cysteinyldopas and indole metabolites as biochemical markers of melanocyte function and malignant melanoma have been facilitated by recent developments in their syntheses and analyses. These investigations revealed that 5SCD urine concentrations in healthy humans increase with exposure to sunlight (27), with individuals of Celtic origin exhibiting the most marked increases (28). Subsequently, exposure to both ultraviolet light in the A region, 400–320 nm (320–280 nm or <280 nm) UVA (29) and UVB (30) light was shown to increase 5SCD serum concentrations in healthy individuals. Additionally, psoriatic patients treated with 8-methoxypsoralen plus UVA (PUVA) therapy (i.e. 8-methoxypsoralen and UVA light) increased serum concentrations of 5SCD and 6HMICA (31,32). The latter metabolite was suggested to mark melanogenic activity more specifically by Rorsman and his colleagues, because albino animals found to contain measurable levels of 5SCD were devoid of 6HMICA (33).

So, the melanocytic amino acid 5SCD and indolic metabolites have been useful biochemical markers of normal and pathological melanocyte function (20). Controlled light exposure or PUVA generally leads to a 100–300% increase in 5SCD serum levels (relative to normal serum levels in the range of 4–16 nmol/l) (29); however, the local concentrations in the skin and especially in the melanocyte may be much higher. From these studies, it may be postulated that a variety of environmental insults may activate melanocytes to cause an increase in both the production of melanin precursors and metabolites.

Melanogenic metabolite photochemistry. UV radiation-induced oxidation of melanogenic catechols, such as that of dopa to dopachrome and ultimately to melanin, first was described by Arnow (34). Felix and Sealy confirmed the purported free radical nature of catecholamine photochemistry spectroscopically by using ESR (35,36). Steady-state electron spin resonance (ESR) spectra of the corresponding o-semiquinones were generated by continuous-wave photolysis of a number of catechols and catecholamines. Because o-semiquinones may arise from catechols, either via photoionization or by phenolic O-H bond homolysis, ESR spin trapping with the spin trap 5,5-dimethyl-1-pyrolline *N*-oxide (DMPO) was employed to scavenge hydrated electrons, hydrogen atoms or both produced during dopa photolysis (37). This study demonstrated that although dopa undergoes both photoionization and bond homolysis upon irradiation at neutral pH, the former predominates over the latter pathway by an estimated 2.6-fold.



SCHEME 2. Formation of methylated melanin metabolites.

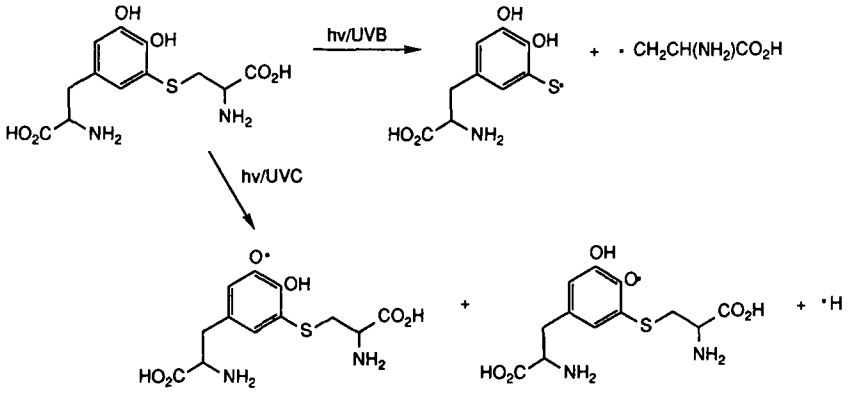
Laser flash photolysis was used to observe directly catechol photoionization for the first time (38). Photoionization of dopa allowed the direct spectroscopic observation of an hydrated electron, indicating the quantum yield for the process to be 9.1%. Absorption spectra, extinction coefficients and rate constants for formation and decay of transients produced upon pulse radiolytic one-electron oxidation of dopa and the cysteinyldopas were used to investigate the photochemistry of the catechols (39,40). Because flash photolysis of dopa yielded a quantum yield of hydrated electron corresponding only to approximately half the yield of dopasemiquinone (19.6%), dopa photoionization and phenolic O-H bond photohomolysis are implicated as being equally efficient processes. The estimated predominance of dopa photoionization over O-H photohomolysis by Kalyanaraman and coworkers is based on an indirect observation of $e^-_{aq}/H^\cdot$ formation, rather than a more accurate, direct observation of e^-_{aq} and dopasemiquinone formation. The rapid disproportionation of dopasemiquinone to form dopaquinone and dopa was demonstrated by Land et al. The subsequent decay of dopaquinone to form dopachrome proceeds via a base-catalyzed unimolecular cyclization (38).

Laser flash photolysis of the pheomelanin precursors 5SCD and DCD yielded fewer hydrated electrons from the cysteinyldopas than from dopa; ($\phi_{photoionization}$ = 1.2% and <0.5%, for 5SCD and DCD, respectively) (38). Markedly different peak wavelengths and spectral shapes were exhibited by the initial flash photolysis transient absorption spectra of 5SCD and DCD compared with semiquinones produced by pulse radiolysis (41). Thus, photohomolytic pathways were implicated as the primary photochemical events in 5SCD photolysis (Scheme 3) (38). Further investigation of these radical pathways have used ESR spin-trapping techniques for continuous wave photolysis experiments.

By continuous wave, 5SCD was found to be about one order of magnitude more photolabile than dopa using 254 nm radiation, and quantum yields for photodestruction were 6.8 ± 0.5% for 5SCD and 0.9 ± 0.1% for dopa (42). Evidence for production of both the hydrated electron and the hydrogen atom was provided by ESR spectra of 280 nm photolysis of dopa in argon- and N_2O-saturated solution of DMPO. These findings corroborate ESR spin trapping results obtained by Kalyaranaman et al. (37) and laser flash photolysis results of Land et al. (38).

Photolysis of 5SCD with 290 nm radiation in the presence of DMPO gave rise to an ESR spectrum unlike dopa and characteristic of a carbon-centered DMPO spin adduct, whereas shorter wavelength light (i.e. 260 nm) afforded a spectrum consisting of the predominant carbon-centered spin adduct as well as the DMPO-hydrogen adduct (42). Because N_2O-saturation did not affect the DMPO-H adduct signal observed during UVC photolysis of 5SCD, the adduct was thought to arise primarily from hydrogen atoms and not the hydrated electron. Model compounds used for further investigations suggested that the carbon-based radical probably did not arise from the dopa alanyl side chain. Subsequently, photolysis of 5SCD in the presence of MNP yielded an ESR spectrum indicative of the MNP-alanyl radical adduct. Thus, the homolytic cleavage of the S-CH_2 bond of the cysteinyl side chain generating both alanyl and thiyl radicals was confirmed (see Scheme 3) (42).

Melanin-like polymeric substances were formed by subjecting dopa or cysteinyldopas to prolonged photolysis. Dopaquinone was produced photochemically from dopa to suggest that the indolic compounds generated by both intramolecular cyclization of the dopaquinone and the tyrosinase-catalyzed polymerization of dopa to eumelanins are similar (43). The photochemical behavior of melanogenic indole intermediates and metabolites did



SCHEME 3. Formation of free radicals following UVB and UVC irradiation of 5SCD.

not receive much attention until recently. De Mol and coworkers investigated the photoproducts formed during catecholamine photolysis (44), and found melanin-like compounds were formed when 5,6-dihydroxy-N-methylindole was irradiated. The investigation of free radical production from DHI in the presence of zinc by Felix and Sealy (36) yielded an ESR spectrum during 254-nm irradiation. The spectrum was assigned to the metal complex of the indolesemiquinone, which suggests that photoionization, phenolic O-H bond homolysis or both may occur in DHI.

Further characterization of the photochemical behavior of the melanogenic indole intermediates and metabolites DHI, DHICA and 6HMICA included a comparison of the relative photolability of the indoles by determination of their quantum yields for destruction by broad-band 300 nm radiation (45,46). The rank order of photolability was found to be DHI > DHICA > 6HMICA ($\phi = 4.9 \pm 0.8\%$, $3.3 \pm 0.3\%$, and $1.9 \pm 0.3\%$, respectively). Continuous-wave photolysis of these indoles monitored by UV-visible difference spectroscopy revealed isosbestic points for all three indoles, which suggests an initial conversion of the indoles to a single photoproduct. The photoproducts of the indole-2-carboxylic acids are stable for minutes to hours before polymerizing, while the analagous DHI photoproducts form highly colored substances much more rapidly.

To characterize further the mechanics of the photochemistry, these melanogenic indoles were studied using ESR spin-trapping techniques (45). Photolysis of DHI with 300-nm radiation in argon- and N_2O-saturated DMPO solutions provided evidence that hydrated electrons and hydrogen atoms were produced. Thus, DHI and dopa seem to have similar photochemistry involving both photoionization and X-H bond homolysis. In contrast, photolysis of DHICA and 6HMICA in the presence of the spin-trap DMPO yielded ESR spectra, which consisted of two spin adducts: the DMPO-hydrogen adduct and a six-line spectrum characteristic of a carbon-centered radical adduct. The DMPO-H adduct was not quenched by N_2O, suggesting that this adduct originates not from the hydrated electron but from the hydrogen atom. Further ESR experiments attempted with several nitrone and nitroso spin trapping reagents were unable to characterize the carbon-based DMPO adducts observed with DHICA and HMICA (45).

The ESR spin trapping experiments detailed above indicate that for DHI, photoionization is an efficient process for DHI but some photohomolysis also may occur, leading to the production of hydrogen atoms. However, the hydrogen atom may be produced via two alternative photohomolytic cleavages on a catecholic indole: O-H bond fission, or N-H bond fission (38,39).

In summary, we have demonstrated that irradiation of a class of endogenous photosensitizers, melanogenic metabolites, gives rise to a number of reactive free radicals. The types of cellular damage promoted by these radicals depend on the rate constants of reaction with cellular biomolecules, the accessibility of these radicals to cellular biomolecules and the concentration of the radicals and biomolecules of interest within a specific cellular environment. While plasma levels of melanogenic metabolites range from 4–16 nmol/l in humans (47), local concentrations in the skin, and particularly in actively pigmenting melanocytes, undoubtedly are much higher. Indeed, we recently have calculated the content of 5-S-cysteinyldopa within actively pigmenting melanoma cells based on data from Rorsman and coworkers (personal communication) and found concentrations to be as high as 200 μM. It is unknown whether melanogenic metabolites localize within intracellular environments; however, it is tempting to speculate that the more hydrophobic methylated metabolites may localize in the lipid portion of biomembranes. Previous studies of light-induced reactions of melanin precursors indicated that several critical cellular components including DNA, proteins and lipids are possible targets (48–51).

The reactivity of the hydrated electrons and the hydrogen atom with various biomolecules is well-known from studies of aqueous radiochemistry. These two radical species have very high reaction rate constants with DNA and various amino acid moieties found in proteins, including aromatic amino acids, the protonated histidyl residue, sulfhydryl and disulfide bonds, as well as the peptide bond carbonyl (52–54). Hydrated electrons and hydrogen atoms also react rapidly with carbon-carbon double bonds such as those found in polyunsaturated fatty acids. In the presence of oxygen, these may lead to long-lived peroxy radicals (55,56). If these photoreactions occur in vivo, our studies suggest a possible role for melanogenic metabolite photolysis in acute solar responses of human skin such as erythema, as well as in sequellae

of chronic solar exposure including premature aging and carcinogenesis.

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REFERENCES

1. Mantagna, W., and Parakkal, P.F. (1974) *The Structure and Function of Skin*, pp. 75-86, Academic Press, New York.
2. Blois, M.S., Fitzpatrick, T.B., Daniels, F. Jr., and Quevedo, W.C. (1969) in *The Biologic Effects of Ultraviolet Radiation* (Urbach, F., ed.) pp. 325-326, Pergamon Press, New York.
3. Blum, H.F. (1961) *Quart. Rev. Bio. 36*, 50-63.
4. Daniels, F. Jr. (1964) in *Handbook of Physiology* (Dill, D.B., ed.) Sec. 4, pp. 969-987, Environmental American Physiological Society, Washington, DC.
5. Daniels, F. Jr. (1974) in *Cancer Epidemiology and Prevention: Current Concepts* (Schottenfeld, D., ed.) pp. 126-152, Charles C. Thomas, Springfield, IL.
6. Johnson, B.E., Daniels, F. Jr., and Magnus, I.A. (1968) in *Photophysiology Current Topics* (Giese, A.C., ed.) Vol. 4, pp. 139-202, Academic Press, New York.
7. Pathak, M.A., and Fitzpatrick, T.B. (1974) *Sunlight and Man* (Pathak, M.A., Harber, L.C., Seiji, M., and Kukita, A., eds.) pp. 725-750, University of Tokyo, Tokyo, Japan.
8. Quevedo, W.C. Jr., Fitzpatrick, T.B., Pathak, M.A., and Jimbow, K. (1975) *Am. J. Phys. Anthrop. 43*, 393-408.
9. Jimbow, K., Fitzpatrick, T.B., and Quevedo, W.C. Jr. (1985 in *Structure and Function of Melanin* (Jimbow, K., ed.) Vol. 2, pp. 71-82, Fuji Printing Co., Sapporo, Japan.
10. Raper, H.S. (1928) *Physiol. Rev. 8*, 245-282.
11. Mason, H.S. (1967) in *Advances in Biology of the Skin, The Pigmentary System* (Montagu, W., and Hu, F., eds.) Vol. 8, pp. 293-312, Pergamon, Oxford, England.
12. Blois, M.S. (1978) *Photobiol. Rev. 3*, 115-134.
13. Ito, S. (1986) *Biochim. Biophys. Acta 883*, 155-161.
14. Prota, G. (1972) in *Pigmentation: Its Genesis and Biologic Control* (Riley, P., ed.) pp. 615-630, Appleton Century Crofts Meridith Corp., New York.
15. Prota, G., and Nicolaus, R.A. (1976) in *The Pigmentary System. Advances in Biology of Skin* (Montagu, W., and Hu, F., eds.) Vol. 3, pp. 323-328, Pergamon Press, Oxford, England.
16. Prota, G., Schenillo, G., and Nicolous, R.A. (1968) *Gazz. Chim. Ital. 98*, 495-510.
17. Agrup, G., Hansson, C., Kennedy, B.M., Persson, K., Rorsman, H., Rosengren, A.M., and Rosengren, E. (1976) *Acta Derm. Venereol. 56*, 491-492.
18. Prota, G., Rorsman, H., Rosengren, A.M., and Rosengren, E. (1977) *Experientia 33*, 720-721.
19. Rorsman, H., Agrup, C., Rosengren, A.M., and Rosengren, E. (1979) *Pigment Cell 4*, 244-252.
20. Rorsman, H., Agrup, G., Hansson, C., and Rosengren, E. (1983) *Pigment Cell 6*, 93-115.
21. Axelrod, J., and Lerner, A.B. (1963) *Biochim. Biophys. Acta 71*, 650-655.
22. Duchon, J., and Matous, B. (1967) *Clin. Chim. Acta 16*, 397-402.
23. Pavel, S., Muskiet, F.A.J., de Ley, L., The, T.H., and van der Silk, W. (1983) *J. Cancer Res. Clin. Oncol. 105*, 275-279.
24. Pavel, S., Elzinga, H., Muskiet, F.A.J., and Wolthers, B.G. (1983) *Acta Derm. Venereol. 63*, 340-343.
25. Pavel, S., Elzinga, H., Muskiet, F.A.J., Smit, J.M., Mulder, N.H., and Schraffordt-Koops, H. (1986) *J. Clin. Chem. Clin. Biochem. 24*, 167-173.
26. Graef, V., and Paul, E. (1982) *Br. J. Derm. 103*, 53-57.
27. Rorsman, H., Agrup, G., Falck, B., Rosengren, A.M., and Rosengren, E. (1976) *Pigment Cell 2*, 284-289.
28. Nixon, P.F. (1978) *Aust. N.Z. J. Surg. 48*, 17-21.
29. Tegner, E., Rorsman, H., and Rosengren, E. (1983) *Acta Derm. Venereol. 63*, 21-25.
30. Tegner, E., Agrup, G., and Rosengren, E. (1983) *Acta Derm. Venereol. 63*, 149-152.
31. Hansson, C., Rorsman, H., Rosengren, E., and Tegner, E. (1981) *Acta Derm. Venereol. 61*, 251-255.
32. Hansson, C., Wirestrand, L.E., Aronsson, A., Rorsman, H., and Rosengren, E. (1985) *Photodermatology 2*, 52-57.
33. Eklund, M.C., Carstam, R., Hansson, C., Rorsman, H., and Rosengren, E. (1985) *Acta Derm. Venereol. 65*, 437-439.
34. Arnow, L.E. (1937) *J. Biol. Chem. 120*, 151-173.
35. Felix, C.C., and Sealy, R.C. (1981) *J. Am. Chem. Soc. 103*, 2831-2836.
36. Felix, C.C., and Sealy, R.C. (1981) *Photochem. Photobiol. 34*, 423-429.
37. Kalyanaraman, B., Felix, C.C., and Sealy, R.C. (1982) *Photochem. Photobiol. 36*, 5-12.
38. Land, E.J., Thompson, A., Truscott, T.G., Subbarao, K.V., and Chedekel, M.R. (1986) *Photochem. Photobiol. 44*, 697-702.
39. Craw, M., Chedekel, M.R., Truscott, T.G., and Land, E.J. (1984) *Photochem. Photobiol. 39*, 115-119.
40. Santus, R., Bazin, M., and Aubailly, M. (1980) *Rev. Chem. Intermed. 3*, 231-283.
41. Thompson, A., Land, E.J., Chedekel, M.R., and Truscott, T.G. (1985) *Biochim. Biophys. Acta 843*, 49-57.
42. Koch, W.H., and Chedekel, M.R. (1986) *Photochem. Photobiol. 44*, 703-710.
43. Chedekel, M.R., Land, E.J., Thompson, A., and Truscott, T.G. (1984) *J. Chem. Soc. Chem. Commun.*, 1170-1172.
44. De Mol, N.J., Beyersbergen van Henegouwen, G.M.J., and Gerritsma, K.W. (1979) *Photochem. Photobiol. 29*, 7-12.
45. Koch, W.H., and Chedekel, M.R. (1987) *Biochim. Biophys. Acta 924*, 458-466.
46. Thompson, A., Koch, W.H., Chedekel, M.R., Land, E.J., and Truscott, T.G. (1986) *J. Invest. Dermatol. 87*, 407.
47. Nimmo, J.E., Hunter, J.A.A., Percy-Robb, I.W., Jay, B., Phillips, C.I., and Taylor, W.O.G. (1985) *Acta Derm. Venereol. 65*, 169-171.
48. Riley, P.A., Sawyer, B., and Wolff, M.A. (1975) *J. Invest. Dermatol. 64*, 86-89.
49. Wick, M.M. (1978) *J. Invest. Dermatol. 71*, 13-164.
50. Moldeus, P., Nordenskjold, M., Bolcsfoldi, G., Eiche, A., Hoglund, U., and Lambert, B. (1983) *Mutation Res. 124*, 9-24.
51. Miranda, M., Botti, D., and Dicol, M. (1984) *Mol. Gen. Genet. 193*, 395-399.
52. Anbar, M., Bambenek, M., and Ross, A.B. (1973) *Natl. Stand. Ref. Data Ser., Natl. Bur. Stand. 43*, 14.
53. Anbar, M., Ross, F., and Ross, A.B. (1975) *Natl. Stand. Ref. Data Ser., Natl. Bur. Stand. 51.*
54. Bensasson, R.V., Land, E.J., and Truscott, T.G. (1983) in *Flash Photolysis and Pulse Radiolysis, Contributions to the Chemistry of Biology and Medicine*, pp. 93-120, Pergamon Press, Oxford, England.
55. Ingold, K.U. (1969) *Acc. Chem. Res. 2*, 1-9.
56. Simic, M. (1973) in *Fast Processes in Radiation Chemistry and Biology* (Adams, G.E., Fielden, E.M., and Michael, B.D., eds.) pp. 162-179, John Wiley and Sons, Bristol, England.

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Reactive Oxygen in the Tumor Promotion Stage of Skin Carcinogenesis[1]

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Exposure of isolated SENCAR mouse epidermal cells to the tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA) in vitro resulted in the production of oxidant species detected as chemiluminescence. This oxidant response can be inhibited by superoxide dismutase and copper complexes but not catalase or scavengers of hydroxyl radical or singlet oxygen, suggesting that the oxidant is superoxide anion. Inhibitors of various parts of the arachidonate cascade affect the TPA-induced oxidant response in a manner that corresponds to their effects on in vivo tumor promotion experiments. Agents that inhibit lipoxygenase activity, i.e. nordihydroguaiaretic acid, benoxaprofen, but not agents that are cyclooxygenase inhibitors, i.e. indomethacin, are effective in suppressing the oxidant response to TPA. Phospholipase C but not phospholipase A_2 or D produced an oxidant response kinetically similar to that elicited by TPA. The inhibitors of TPA-induced oxidants inhibited the phospholipase C response to the same extent, suggsting that TPA and phospholipase C may produce an oxidant species through a common mechanism, via phospholipid turnover-protein kinase C activation. The relevance of oxidant production to the tumor promotion process is suggested by the ability of exogenous xanthine/xanthine oxidase, a superoxide anion-generating system, to induce ornithine decarboxylase, a characteristic of TPA-treated cells. In addition, oxidant production is significantly lower in cells from the TPA-promotion resistant C57BL/6J mouse. These studies provide further support for a role for reactive oxygens in the tumor promotion process.
Lipids 23, 592-597 (1988).

Experimental chemical carcinogenesis studies in animals are valuable in identifying those biological events or agents that play either an essential or modulatory role in the development of tumors. Mouse skin has proven to be one of the best model systems for studying the multistage nature of carcinogenesis (1-3). Skin tumors can be induced readily by the sequential application of a subthreshold dose of carcinogen, operationally referred to as the initiation stage, followed by repetitive treatment with a noncarcinogenic tumor promoter (promotion stage). This second stage, promotion, most often is accomplished by using the phorbol diester 12-0-tetracecanoylphorbol-13-acetate (TPA), although a variety of agents have been identified as skin tumor promoters, including benzoyl peroxide, dihydroteleocidin, anthralin and its derivative chrysarobin (4,5).

The mode of action of TPA has been under investigation for numerous years, but elucidation of its mechanism(s) has been hampered in part by TPA, as well as other promoters, causing a plethora of morphological and biochemical responses in the skin (4,6). One of the prominent responses, inflammation and vascular permeability changes, (7) have provided the basis for inquiry into the role of mediators of these events in the promotion process. The particular mediators under investigation by this and other laboratories are arachidonic acid metabolites (8-11) and activated oxygen species (12).

Substantial evidence (reviewed in Ref. 12) that suggests that the generation of free radicals, such as superoxide anion and hydroxyl radical, may be involved in tumor promotion has been accumulating in the last few years. The most direct evidence comes from the work of Slaga et al. (13) in which free radical generating compounds, such as benzoyl peroxide, lauroyl peroxide and chlorperbenzoic acid were shown to be complete tumor promoters. Indirect evidence comes from studies demonstrating that various antioxidants are inhibitors of TPA tumor promotion; butylated hydroxyanisole (14), vitamin E and vitamin C (15), dimethylsulfoxide (14) and the superoxide dismutase (SOD) mimetic copper II diisopropylsalicylic $acid)_2$ (Cu DIPIS) (16) have been shown by Kensler and Trush (17) and Kozumbo et al. (18) to inhibit tumor promotion. Butylated hydroxyanisole and CuDIPS also have been reported to inhibit TPA-induced ornithine decarboxylase (ODC) activity. In addition, Solanki et al. (19) found that application of TPA to mouse skin resulted in a substantial decrease in the endogenous levels of SOD and catalase, the major detoxification enzymes for superoxide and H_2O_2 respectively. Numerous in vitro studies have been performed as well, particularly with polymorphonuclear leukocytes, on the generation of excited oxygen species by TPA (20-24).

These studies raised the question of whether TPA-induced oxidant generation in the skin is attributable to the inflammatory cell infiltration of the dermis or if TPA can induce an oxidant response in epidermal cells as well. Our laboratory has undertaken to address this question and the sequel issues of the identity, metabolic source and biological activity of the oxidants(s), and the essential nature of oxidant production to the tumor promotion process. This paper describes the approaches that we have taken to these problems and presents the results of investigations (11,25-27) and on-going studies.

METHODS

Animals. Female and male SENCAR mice were obtained from NCI (Frederick, MD). SSIN (inbred SENCAR) mice are bred at this facility. Male and female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME).

All strains of mice were bred at this facility to minimize any influences due to differences in diet and environment.

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Abbreviations: CL, chemiluminescence; CuDIPS, copper II (3,4-diisopropylsalicylic $acid)_2$; HA-1004, N-(2-quanidinoethyl)-5-isoquinoline sulfonamide HCl; H-7; 1-(5-isoquinoline sulfonyl)-2-methyl piperazine dihydrochloride; ODC, ornithine decarboxylase; SOD, superoxide dismutase; TPA, 12-0-tetradecanoylphorbol-13-acetate.

Newborn mice from these parental stocks were used in these studies.

Chemiluminescence (CL) measurements. Epidermal cells were isolated from the newborn mice by trypsinization (24). The absence of leukocyte contamination was monitored as described (25). Chemiluminescence of 1×10^7 epidermal cells was measured as described (25) using an LKB Rackbeta Model 1218 ambient temperature liquid scintillation counter set in the CL mode. The assay was carried out in 7-ml glass minivials by adding the cells to 3 ml of Dulbecco's phosphate-buffered saline containing 5 mM glucose and 1 μg/ml luminol. After determining the background CL of each vial, solvent or test agent was added to start the reaction. The CL was monitored continuously for up to 30 min at 10-sec intervals. Either saline, ethanol or dimethylsulfoxide was used as the solvent for all the agents; due to the quenching activity of the latter two, the amount used was never more than 10 μl per 3-ml assay. Results are expressed as counts/unit time and the data expressed as either peak response or temporal curves.

ODC activity. Twenty-four-hr-old primary cultures of epidermal cells were treated for seven hr with either 1 μg/ml TPA and/or inhibitors at the given doses, 0.1 units/ml phospholipase C and/or inhibitors at the given doses or 10 μg/ml xanthine and 0.45 units/ml xanthine oxidase and/or inhibitors at the given doses. The medium then was discarded and a 12,000 × g centrifugation performed following homogenization of the scraped cells. The supernatant was used to determine enzyme activity measured as the release of $^{14}CO_2$ from [^{14}C]-L ornithine, as described (28) and the total protein measured by the Bio-rad Coomassie blue reaction.

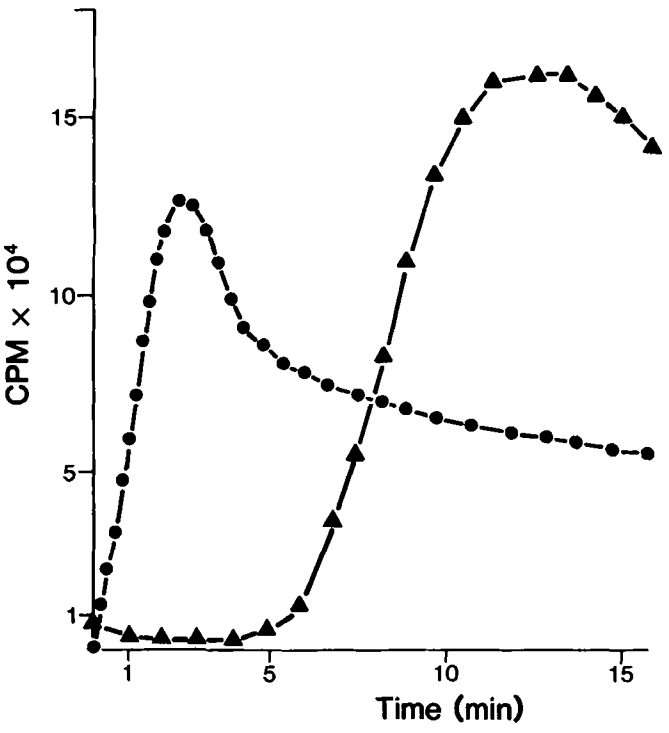

FIG. 1. Comparison of the chemiluminescence response to TPA of human PMN and mouse epidermal cells. Epidermal cell assay required the use of luminol, ●, 100 ng/TPA added to 10^7 PMN; ▲, 100 ng/ml TPA added to 10^7 epidermal cells. No response seen with solvent controls (acetone).

RESULTS AND DISCUSSION

TPA-induced oxidant generation in epidermal cells. One of the major obstacles in the detection of reactive oxygen species in nonleukocytes is the lack of sensitivity of available methods or assays for such detection. For this reason, these studies on epidermal cells used a modification (25) of a chemiluminescence (CL) assay described by Kensler et al. (21) for measuring reactive oxygen production in polymorphonuclear leukocytes. The chemiluminescence enhancer luminol is an absolute requirement for the assay for epidermal cells but not for polymorphonuclear leukocytes, suggesting that the level of oxygen radicals produced is considerably less (approximately 10,000-fold) than in leukocytes. The extremely low level of oxidant production by the epidermal cells probably accounts for the negligible cytochrome C reduction seen in that assay (25). The CL response of epidermal cells to TPA, shown in Figure 1, also is different kinetically from that seen with polymorphonuclear leukocytes. The epidermal cell response to TPA produces a distinct rise after five min, rapidly reaches a peak by 15 min and slowly tapers off over the next 30 min or more. The magnitude of the response correlates with the dose of TPA as well as the number of cells used in the assay (25). In addition, a series of phorbol esters with different promoting abilities produced corresponding CL responses (25).

Oxidant identification. The approach taken to identify the oxidant species responsible for the CL response to TPA was to include in the assay known scavengers with specificites for particular reactive species. For example, the contribution of the superoxide anion to CL may be direct or indirect (29), but an indication of its participation can be determined through the use of SOD, an enzyme specific for the dismutation of this radical (29). As shown in Figure 2, inclusion in the CL assay of either SOD, the SOD-mimetic CuDIPS or $CuSO_4$ nearly negated the TPA-induced CL response. On the other hand, neither catalase, which breaks down H_2O_2, nor mannitol or sodium benzoate, scavengers of hydroxy radicals, inhibited CL to any appreciable extent. The singlet oxygen scavengers histidine or diazobicyclooctane also had no suppressive effect. These data suggest that the species responsible for the majority of the observed CL response is superoxide anion. Because the CL assay measures extracellular or cell-membrane surface oxidants and because SOD presumably does not enter the cell, it is concluded that the superoxide is generated in the membrane.

Metabolic source. The insertion of molecular oxygen into arachidonic acid and subsequent rearrangements and metabolism produce reactive oxygen species and the hydroperoxides of the fatty acid. Because epidermal cells have been shown to produce prostaglandins and other arachidonate metabolites in response to TPA, it was anticipated that inhibitors of various parts of the arachidonate cascade would affect the magnitude of the TPA-induced oxidant response. As indicated in Table 1 and described in detail by Fischer and Adams (25), the effect of these inhibitors depends upon the particular pathway against which they are effective. Those inhibitors that are

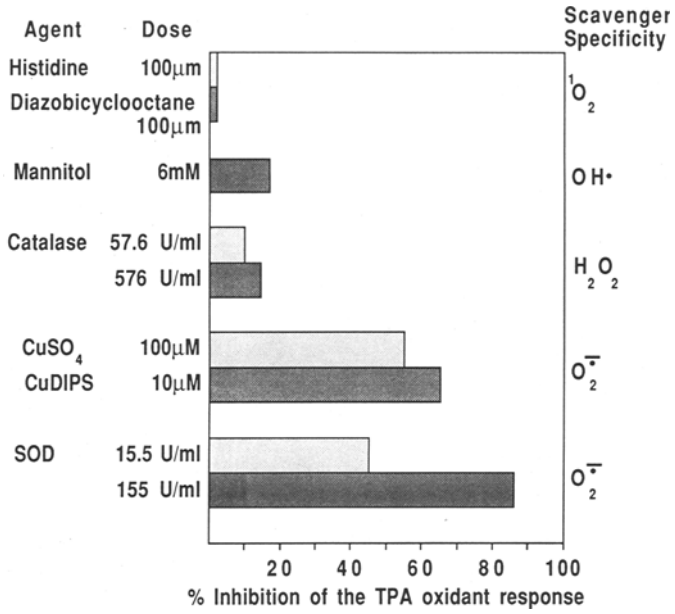

FIG. 2. Effect of free radical modifiers on the chemiluminescence of TPA-treated epidermal cells. 10^7 epidermal cells were used in each assay; the cpm at the peak of the response were used to determine the percentage of the TPA-alone response.

TABLE 1

Effect of Inhibitors of Arachidonic Acid Metabolism on TPA-stimulated Chemiluminescence in SENCAR Epidermal Cells

Agent	Dose (μM)	% TPA CL response	Effect on promotion
Cyclooxygenase inhibitors			
Indomethacin	1	99	Enhance
	10	106	
	100	93	
Flurbiprofen	1	110	Enhance
	10	93	
	100	79	
Lipoxygenase/cyclooxygenase inhibitors			
Phenidone	1	42	Inhibit
	10	26	
	100	0	
Benoxaprofen	33	35	Inhibit
	100	23	
	300	2	
Quercetin	10	76	Inhibit
	100	26	
Nordihydroguairaretic acid	1	65	Inhibit
	10	0	
ETYA	10	84	Inhibit
	100	9	

Chemiluminescence assay performed as described (25). Tumor promotion data taken from Refs. 9, 30–33.

predominantly lipoxygenase inhibitors, such as benoxaprofen, or inhibit both cyclooxygenase and lipoxygenase, such as phenidone, are effective in diminishing the TPA-induced CL response. However, cyclooxygenase inhibitors, i.e. indomethacin, are not inhibitory. This data correlates well with both their effect on tumor promotion (9,30,33) and their effect on TPA-induced DNA synthesis in vivo and in vitro (34).

Because the above study suggested that a source of the TPA-stimulated oxidant was arachidonic acid metabolism via the lipoxygenase pathway, the question arose as to the mechanism of arachidonate release from phospholipids. Free arachidonate can result from either phospholipase A_2 activity or phospholipase C activity followed by diacylglycerol lipase action. This latter pathway is of particular interest because TPA both competes for diacylglycerol binding and activation of protein kinase C, as described by Nishisuka (35), and can increase diacylglycerol production in mouse epidermal cells through what is presumed to be phospholipase C activity.

To determine whether a particular phospholipase activity is involved in the TPA-induced CL response, different types of phospholipases were compared for their ability to induce CL in mouse epidermal cells. As shown in Figure 3, and described by Fischer et al. (26), a CL response can be induced only by phospholipase C and most notably when this enzyme is isolated from *Clostridia perfringens.* Kinetically, this response differs from TPA-induced CL in that it typically occurs several minutes earlier than the TPA response. Concomitant treatment with TPA and phospholipase C results in a partial, but not complete, additivity. The phospholipase C response is suppressed, and to nearly the same extent, by the same agents that inhibit the TPA response (26). Because neither exposure

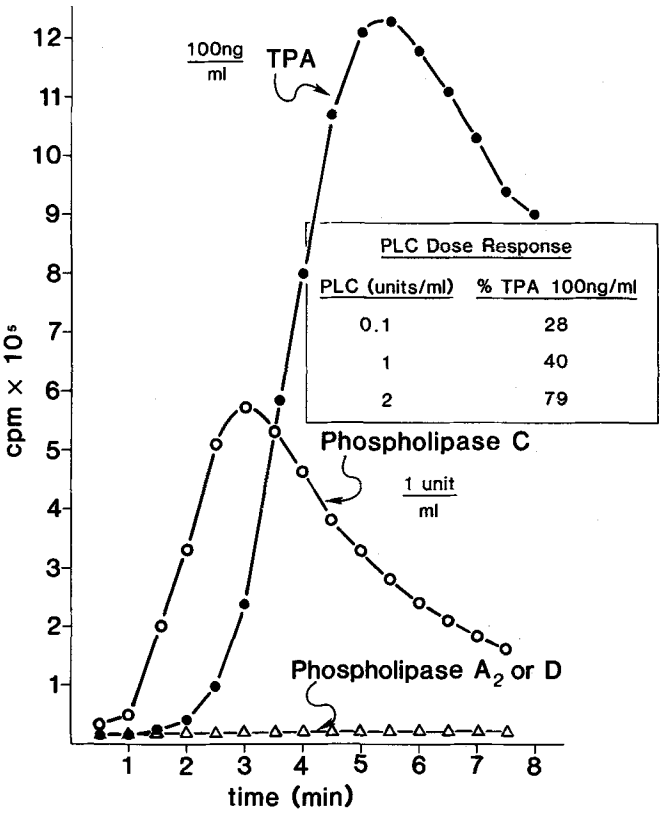

FIG. 3. Comparison of different phospholipases in their ability to induce chemiluminescence in mouse epidermal cells. Phospholipases at doses of 1–100 units/ml were included in the assay (in the absence of TPA) as possible chemiluminescence inducing agents.

to phospholipase A_2 (from any source) or exogenous arachidonic acid (with or without TPA) results in a CL response, an argument can be made against arachidonic acid metabolism as the source of the oxidant. Previous work (data not presented) has demonstrated that epidermal cells readily metabolize exogenous arachidonate and both phospholipase A_2 and C treatment results in the release of arachidonate from prelabeled epidermal cells. Because only phospholipase C produces a CL response, it appears that the oxidant is generated through the phospholipid-diacylglycerol-protein kinase C pathway, shown in Figure 4. It is possible that protein kinase C activates oxidative enzymes such as NADPH oxidase and that this or other oxidases are the source of the superoxide anion. This suggestion stems from the demonstration that in stimulated inflammatory cells such as neutrophils, NADPH oxidative metabolism is a source of reactive oxygens, particularly superoxide anion (22).

To pursue this hypothesis, studies with protein kinase C inhibitors were carried out using both the CL assay and the induction of ornithine decarboxylase (ODC), a known protein kinase C-mediated event (36). Two pairs of compounds were selected such that each was comprised of a protein kinase C inhibitor and its reportedly inactive analog, used to control for any nonspecific effects (37, 38). With the first pair, the inhibitor palmitoylcarnitine (100 μM) inhibited TPA-induced ODC by 50% and phospholipase C-induced ODC by 74% as indicated in Table 2. The negative analog acetylcarnitine was without activity. With the second pair of compounds, the inhibitor H-7 (100 μM) completely inhibited both TPA and phospholipase C-induced ODC; at the same dose, the negative analog HA-1004 had no effect. When these agents were included in the CL assay, similar results were seen: 100 μM palmitoylcarnitine or H-7 but not acetylcarnitine or HA-1004 almost completely suppressed the CL response to either TPA or phospholipase C. These results suggest that the oxidant response to both TPA and phospholipase C is mediated, at least in part, by protein kinase C.

TABLE 2

Effects of Protein Kinase C Inhibitors on TPA- and Phospholipase C-induced Chemiluminescence and Ornithine Decarboxylase Activity

Inducing agent	Inhibitor (dose, μM)	% Inhibition of CL	% Inhibition of ODC
TPA	H-7 (10)	44	N.D.
	H-7 (50)	N.D.	46
	H-7 (100)	95	100
	HA-1004 (100)	0	0
	PC (10)	30	N.D.
	PC (100)	100	60
	AC (100)	13	0
Phospholipase C	H-7 (10)	27	20
	H-7 (100)	100	100
	HA-1004 (100)	0	0
	PC (10)	65	N.D.
	PC (100)	100	98
	AC (100)	0	20

Mouse epidermal cells were isolated and used either directly in the chemiluminescence (CL) assay or cultured and used in the ornithine decarboxylase (ODC) assay.

H-7, 1-(5-isoquinoline sulfonyl)-2-methylpiperazine dihydrochloride; HA-1004, N-(2)guanidinoethyl)-5-isoquinoline sulfonamide HCl; PC, palmitoylcarnitine; AC, acetylcarnitine; N.D., not determined.

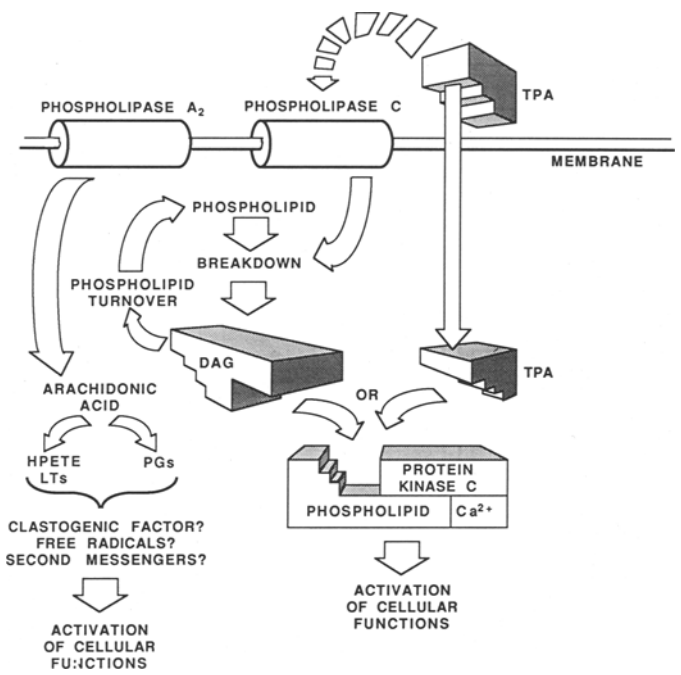

FIG. 4. Relationship of TPA and its protein kinase C receptor to phospholipid metabolism and arachidonate release.

Biological activity of superoxide anion. Two approaches were taken to determine whether superoxide anion production in response to TPA plays a role in subsequent cellular processes. If particular TPA-induced events are mediated by oxidants, specific scavengers or antioxidants should diminish these responses; in addition, exogenous addition of oxidants such as superoxide anion to epidermal cells should elicit some of these events. The event chosen for study was the induction of ODC, believed to be important in tumor promotion since inhibition of this induction is associated with inhibition of promotion (4,6). The work of Kensler et al. (16) in which the SOD mimetic CuDIPS was shown to inhibit TPA-induced ODC, suggested that superoxide is involved in ODC induction. To test this more directly, we treated cultured epidermal cells with xanthine/xanthine oxidase, a superoxide anion-generating system, in the presence or absence of SOD. As indicated in Figure 5, xanthine/xanthine oxidase induced ODC significantly, although only to about half of the level that can be achieved with TPA. Inclusion of SOD inhibited either xanthine/xanthine oxidase- or TPA-induced ODC by approximately 50%. These results suggest that oxidants generated in response to TPA treatment partially mediate the induction of ODC. The lack of a more complete inhibition may be due to the presence of further metabolites of superoxide, specifically H_2O_2. It is not known if a combination of catalase and SOD would produce a more substantial inhibition.

Oxidants and tumor promotion. To determine whether the magnitude of the TPA-induced oxidant response is

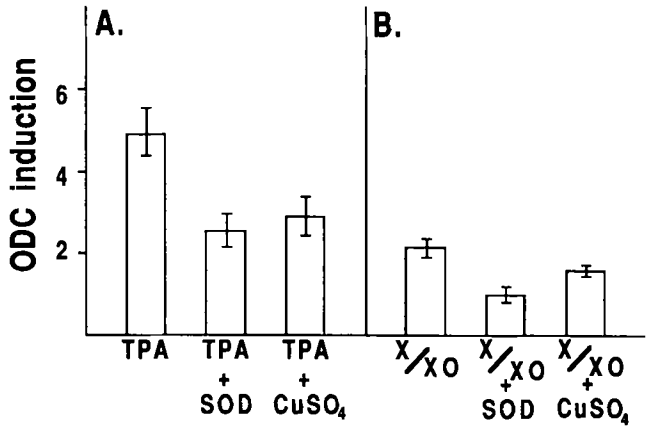

FIG. 5. Effects of superoxide anion dismutating agents on either TPA or xanthine/xanthine oxidase (x/xo) induced ornithine decarboxylase (ODC) induction. ODC activity measured as net nmol CO_2/mg protein/hr was determined seven hr after treatment with either 1 μg/ml TPA (panel A) or 60 μg/ml xanthine plus 0.45 units/ml xanthine oxidase (panel B) in the presence or absence of 155 units/ml SOD or 250 μM $CuSO_4$. Treatment groups of six to eight epidermal cultures were used to determine the mean ± s.e.m. Inhibition by SOD and $CuSO_4$ is significant ($p < .01$, Student's t-test).

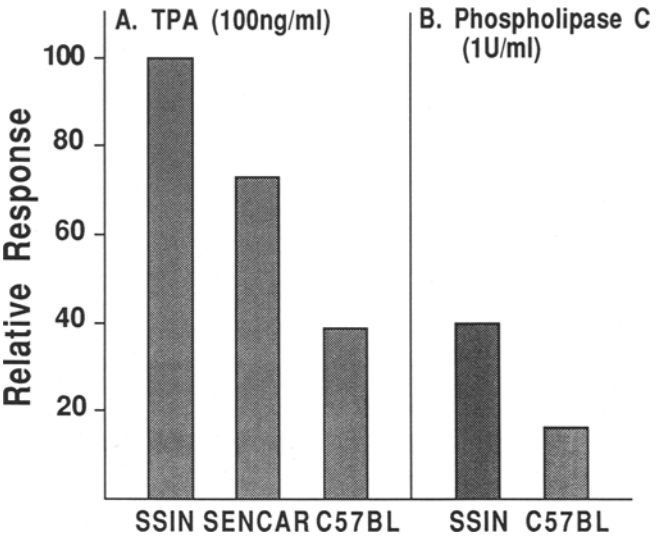

FIG. 6. Comparison of the chemiluminescence response in epidermal cells from SSIN, SENCAR and C57BL/6J mice. Epidermal cells (10^7) from each of the strains of mice were stimulated with either 100 ng/ml TPA (panel A) or 1 unit/ml phospholipase C in the chemiluminescence assay. The peak response, SSIN treated with TPA, was used to determine relative responses of the other groups. The experiments were repeated two to three times and the replicate variation within an experiment was <15%.

relevant to the tumor promotion process, several strains of mice with known sensitivities to TPA as a promoter were compared. Of particular interest is the difference in sensitivity to promotion between SENCAR and C57BL/6J mice: the latter essentially are refractory to TPA promotion, although sensitive to benzoyl peroxide promotion (39). Therefore, it was hypothesized that while the C57BL/6J are able to respond to radicals, TPA may not induce radical production to the same extent as in TPA-sensitive strains of mice. In addition to the above mice, the SSIN, an inbred strain of SENCAR that is more sensitive to TPA (40), also were compared. As shown in Figure 6 and described by Fischer et al. (27), the mice can be ranked SSIN > SENCAR > C57BL/6J on the basis of the magnitude of the CL response to TPA. Lewis and Adams (41,42) have shown that arachidonic acid metabolism and H_2O_2 release from macrophages of SENCAR is greater than from C57BL/6 mice and that the dermal inflammatory response to TPA is greater in SENCAR mice. These correlations between TPA promotion sensitivity and oxidant generation argues in favor of a role for reactive oxygens in the promotion process.

The above studies indicate that the tumor promoter TPA can cause the production of reactive oxygen species, principally superoxide, in mouse epidermal cells. The oxidant is believed to be superoxide and may at least partially mediate some of the effects of TPA. The generation of the same oxidant by phospholipase C suggests that the phospholipid turnover-protein kinase C signaling mechanism may be involved. This is further supported by the finding that specific protein kinase C inhibitors suppress both TPA and phospholipase C induced oxidant response and induced ornithine decarboxylase activity. A correlation was observed between strains of mice with respect to TPA-induced oxidant response and tumor promotion sensitivity. Collectively, these data suggest a strong link between the generation of an oxidant species and at least some aspects of tumor promotion. Clearly, more work is needed to determine the mechanisms involved (i.e., altered redox status in the cell, oxidation of molecules including DNA) and the extent to which oxidant generation is essential to tumor promotion.

ACKNOWLEDGMENTS

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REFERENCES

1. Berenblum, I., and Shubik, P. (1947) *Br. J. Cancer 1,* 373-391.
2. Slaga, T.J., Fischer, S.M., Nelson, K., and Gleason, G.L. (1980) *Proc. Natl. Acad. Sci. USA 77,* 3659-3663.
3. Slaga, T.J., Fischer, S.M., Weeks, C.E., and Klein-Szanto, A.J.P. (1981) in *Rev. Biochem. Tox. (Hodgson, E., Bend, J.R., and Philpot, R.M., eds.) pp. 231-282, Elsevier/North Holland Publishing, New York.*
4. *Slaga, T.J. (1984) in Mechanisms of Tumor Promotion* (Slaga, T.J., ed.) pp. 1-16, CRC Press, Boca Raton, FL.
5. DiGiovanni, J., Decina, P.C., Pritchett, W.P., Cantor, J., Aalfs, K.K., and Coombs, M.M. (1985) *Cancer Res. 45,* 2584-2589.
6. Slaga, T.J., Fischer, S.M., Weeks, C.E., and Klein-Szanto, A.J.P. (1980) in *Biochemistry of Normal and Abnormal Epidermal Differentiation* (Seije, M., and Bernstein, I.A., eds.) pp. 19-218, University of Tokyo Press, Tokyo.
7. Janoff, A., Klassen, A., and Troll, W. (1970) *Cancer Res. 30,* 2568-2671.
8. Furstenberger, G., and Marks, F. (1982) in *Symp. on Cocarcinogenesis and Biological Effects on Tumor Promoters* (Hecker, E., Fusenig, N.E., Kunz, W., Marks, F., and Thielmann, H.W., eds.) pp. 325-330, Raven, New York.
9. Furstenberger, G., and Marks, F. (1985) in *Arachidonic Acid Metabolism and Tumor Promotion* (Fischer, S.M., and Slaga, T.J., eds.) pp. 49-72, Martinus Nijhoff, Boston.
10. Fischer, S.M. (1983) in *Mechanisms of Tumor Promotion* (Slaga, T.J., ed.) Vol. 2, pp. 113-126, CRC Press, Boca Raton, FL.

11. Fischer, S.M. (1985) in *Arachidonic Acid Metabolism and Tumor Promotion* (Fischer, S.M., and Slaga, T.J., eds.) pp. 22–48, Martinus Nijhoff, Boston.
12. Kensler, T.W., and Taffee, B.G. (1986) *Adv. Free Radical Biol. Med. 2,* 347–387.
13. Slaga, T.J., Klein-Szanto, A.J.P., Triplett, L.L., and Yotti, L.P. (1981) *Science 213,* 1023–1025.
14. Slaga, T.J., Fischer, S.M., Weeks, C.E., Nelson, K., Mamrack, M., and Klein-Szanto, A.J.P. (1980) in *Carcinogenesis: A Comprehensive Survey* (Slaga, T.J., ed.) Vol. 5, pp. 19–34, Raven Press, New York.
15. Chan, J.T., and Black, H.S. (1978) *Experentia 34,* 110–111.
16. Kensler, T.W., Bush, D.M., and Kozumbo, W.J. (1983) *Science 221,* 75–77.
17. Kensler, T.W., and Trush, M.A. (1983) *Biochem. Pharmacol. 32,* 3485–3487.
18. Kozumbo, J.L., Seed, J.L., and Kensler, T.W. (1983) *Cancer Res. 43,* 255–2559.
19. Solanki, V., Rana, R.S., and Slaga, T.J. (1981) *Carcinogenesis 2,* 1141–1146.
20. Goldstein, B.D., Witz, G., Amoruso, M., Stone, D.S., and Troll, W. (1981) *Cancer Lett. 11,* 257–262.
21. Kensler, T.W., and Trush, M.A. (1981) *Cancer Res. 41,* 216–222.
22. Trush, M.A., Wilson, M.E., and Van Dyke, K. (1978) *Methods Enzymol. 57,* 462–494.
23. DeChatelet, L.R., Shirley, P.S., and Johnston, R.B. Jr. (1976) *Blood 47,* 545–554.
24. Yuspa, S.H., and Harris, C.C. (1974) *Exp. Cell Res. 86,* 95–105.
25. Fischer, S.M., and Adams, L.A. (1987) *Cancer Res. 45,* 3130–3136.
26. Fischer, S.M., Baldwin, J.K., and Adams, L.M. (1985) *Biochem. Biophys. Res. Commun. 131,* 1103–1108.
27. Fischer, S.M., Baldwin, J.K., and Adams, L.M. (1986) *Carcinogenesis 7,* 915–918.
28. Weeks, C.E., and Slaga, T.J. (1979) *Biochem. Biophys. Res. Commun. 91,* 1488–1496.
29. Bors, W., Suran, M., and Michel, C. (1983) in *Superoxide Dismutase* (Oberley, L.W., ed.) Vol. 2, pp. 31–63, CRC Press, Boca Raton, FL.
30. Fischer, S.M., Gleason, G.L., Mills, G.D., and Slaga, T.J. (1980) *Cancer Lett. 10,* 343–350.
31. Fischer, S.M., Mills, G.D., and Slaga, T.J. (1982) *Carcinogenesis 3,* 1243–1245.
32. Nakadate, T., Yamamoto, S., Iseki, H., Sonoda, S., Takemura, S., Ura, A., Hosoda, Y., and Kato, R. (1982) *Gann 73,* 841–843.
33. Kato, R., Nakadate, T., and Yamamoto, S. (1984) *Carcinogenesis 4,* 1301–1305.
34. Fischer, S.M., Furstenberger, G., Marks, F., and Slaga, T.J. (1987) *Cancer Res. 47,* 3174–3179.
35. Nishisuka, Y. (1984) *Nature 308,* 693–698.
36. Verma, A.K., Pong, R-C., and Erickson, D. (1986) *Cancer Res. 46,* 6149–6155.
37. Wise, B.C., Glass, D.B., Chou, C-H.J., Raynor, R.L., Katoh, N., Schatzman, R.C., Turner, R.S., Kibler, R.F., and Kuo, J.F. (1982) *J. Biol. Chem. 257,* 8489–8495.
38. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) *Biochemistry 23,* 5036.
39. Reiners, J.J., Nesnow, S., and Slaga, T.J. (1984) *Carcinogenesis 5,* 301–307.
40. Fischer, S.M., O'Connell, J.F., Conti, C.J., Tacker, K.C., Fries, J.W., Patrick, K.E., Adams, L.M., and Slaga, T.J. (1987) *Carcinogenesis 8,* 421–424.
41. Lewis, J.C., and Adams, D.O. (1986) *Cancer Res. 46,* 5696–5700.
42. Lewis, J.C., and Adams, D.O. (1987) *Carcinogenesis 8,* 889–898.

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METHODS

Biopsy Method for Human Adipose with Vitamin E and Lipid Measurements

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An adaptation of the needle biopsy procedure of Beynen and Katan for human adipose tissue, which yields 2–10 mg adipose samples, is described and evaluated. Micromethods are presented for the analysis of α-tocopherol, cholesterol and fatty acids in each adipose specimen. The needle biopsy procedure, which uses a Vacutainer to create suction, is compared with a punch biopsy method. The needle biopsy is rapid (6 samples/hr), simple and unobjectionable to the subjects, and provides samples with reproducible ratios of cholesterol and α-tocopherol. Unlike the punch biopsy, the needle biopsy reliably obtains specimens with a lipid composition typical of adipocytes. The needle biopsy method is adaptable to nutritional studies of tocopherol and fatty acid metabolism in adipose, and to studies of hazardous compounds stored in adipose. The linoleic acid content of adipose from residents of the West Coast was found to be considerably higher than values reported earlier. The adipose fatty acid data indicate an increase in human adipose linoleate when compared with earlier reports and suggest a trend toward increasing linoleic acid in the American diet.
Lipids 23, 598–604 (1988).

Evaluation of long-term nutritional status using adipose tissue biopsy has definite advantages for several nutrients over measurement of plasma levels. The adipose levels may provide a useful indication of a long-term average intake for many fat-soluble nutrients. Plasma levels of both unsaturated fatty acids (1) and α-tocopherol (2,3) change very rapidly in humans following change in dietary intake, reaching a new steady-state level in a few days to a week. However, adipose fatty acids in humans take several years to reach new steady-state following dietary change (1,4). Adipose α-tocopherol levels have been shown to respond slowly to dietary change in experimental animals (5,6). Other compounds with a slow turnover in adipose tissue include certain pesticides (7) and halogenated hydrocarbons (8), and adipose levels may indicate effective exposure to many fat-soluble toxic compounds.

There has been considerable interest in measuring adipose tocopherols in human subjects. McMasters et al. (9,10) carried out an analysis of a large number of subjects and examined the effects of dietary α-tocopherol supplements. However, their vitamin E-analysis method had low sensitivity and necessitated taking ca. 1 g surgical fat samples, which is not practical for many studies. Bieri and Poukka Evarts (11) measured tocopherols in autopsy fat, and this work also employed large adipose samples.

More recently, Kayden et al. (12,13) carried out investigations of tocopherol metabolism in human adipose and established that human adipose tocopherol levels are depressed in abetalipoproteinemia and other malabsorptive states. These workers also carried out small-scale trials of the effects of dietary tocopherol supplements on adipose α-tocopherol levels. Kayden et al. utilized the original needle biopsy procedure of Hirsch et al. (1) and used sensitive HPLC techniques for tocopherol measurements.

Needle-biopsy procedures are preferred to more invasive surgical or punch procedures because of speed, lack of discomfort and absence of any suture or scar afterward. Newer microchemical techniques using HPLC or GC/MS have the sensitivity needed to carry out biochemical measurements on the small samples provided by needle biopsy. To facilitate long-term studies of α-tocopherol turnover in human adipose, a simplification of the recently published needle biopsy procedure of Beynen and Katan (14) was developed and tested. An earlier version of this method also was described by Christakis (15). Micromethods to measure tocopherols and the lipid denominators cholesterol and fatty acids also were developed to utilize the milligram adipose samples obtained.

EXPERIMENTAL

Adipose biopsy. Several methods of obtaining human adipose fat were explored. The first approach was the needle biopsy method as originally described by Hirsch et al. (1). This method, which uses saline injection and a large hypodermic syringe to create suction, was slow and fatiguing to use and frequently yielded only 0.5 mg of adipose or less, which is insufficient for the biochemical analyses described in this report. While it may have been possible to improve the performance of the Hirsch method with practice, a method that could be more easily mastered was desired.

A conventional skin biopsy punch (16), using a small 2-mm punch, also was evaluated for adipose sampling because it is easy to obtain specimens of 2 mg or more. Specimens initially were determined to be "fat" by visual observation. About 20 specimens were collected with this method and analyzed. However, it was found that about one-half of the specimens contained virtually no triglyceride. A 22.5-power dissecting microscope then was used to examine specimens in more detail. Many specimens were observed through the microscope and found to consist of a large cluster of fat cells, with pieces of skin or connective tissue adhering to some specimens. The nonadipose tissue was removed by dissection, and the remaining cluster of cells (with a bunch-of-grapes appearance) was thought to be pure fat. This method was easy and rapid but not quite satisfactory. The dissected punch biopsies usually provided adequate triglyceride, but the specimens frequently contained cholesterol far in excess of the normal range for adipocytes (1–2 μg cholesterol/mg triglyceride) (17), which indicated skin contamination.

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Abbreviations: BHT, butylated-hydroxy-toluene; TMS, tetramethylsilane; GC, gas chromatography; HPLC, high performance liquid chromatography.

Finally, the needle biopsy method of Beynen and Katan (14) was used. This method uses a Vacutainer to create suction. With a Vacutainer adapter, we found that the special needle specified by Beynen and Katan was not required, and any standard hypodermic needle could be used. In the work described here, a 1.5-inch, 16-gauge needle, a Vacutainer adapter (American Scientific Products #B3035-10, McGaw Park, IL), a Vacutainer holder, and a 20-cc "red-top" (no anticoagulant) Vacutainer are employed to obtain the adipose biopsies. The sampling area is infiltrated with 1–2 ml of Xylocaine (1% containing 1:100,000 epinephrine), using a 27-gauge needle. The 16-gauge biopsy needle is inserted at a 45-degree angle into the anaesthetized subcutaneous fat pad, just below the dermal interdigitation, and the Vacutainer is engaged. The needle is moved forward and backward 1–2 cm to disrupt fatty tissue. This motion is continued as the entire unit is slowly withdrawn from the skin. If blood oozes from the puncture site, hemostasis is accomplished by a pressure dressing. Otherwise it is simply covered with a Band-aid for 24–48 hr. No visible scar develops, and the test site is recognizable only by a very slight alteration in normal pigmentation at two wk. Two months after the test, the site is no longer visible.

Beynen and Katan did not use anaesthetic in their needle biopsy procedure (14), whereas our subjects found that local anaesthetics were highly desirable.

Usually the fat is lodged into the upper part of the Vacutainer adapter and is collected by back-flushing the adapter with saline into a dish (Fig. 1). By this means, 2–10 mg of fat typically is collected. Repeat samples usually can be collected through the same skin puncture.

When upper-arm and waist adipose tissue are sampled with this procedure, a more shallow needle angle (20–30 degrees) is employed so that the superficial area of subcutaneous fat is sampled. These sites are somewhat more difficult to biopsy but give satisfactory and reproducible results.

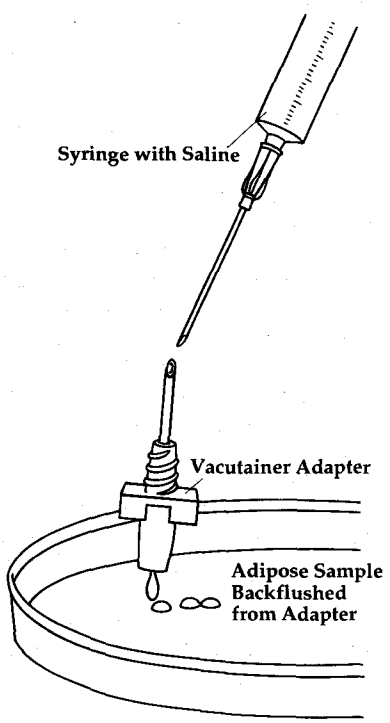

FIG. 1. Technique of recovering adipose biopsy samples from the hub of the Vacutainer adapter, where it usually is lodged.

In the data given in this study, replicate samples (within-day and between-day) always were taken from the same site on each subject.

Human subjects. Healthy adult males, ages 21–60, were recruited for this study from the San Francisco Bay area. An informed consent procedure was followed. The protocol was approved by the human subjects committee, University of California School of Medicine, San Francisco, CA. The subjects were instructed to adhere to their normal diets for the period of the study. Seven of the nine subjects in this study were administered a food-frequency questionnaire to determine dietary intake of lipids as considered in the discussion. The questionnaire used recently was validated by Willett et al. (18).

Reagents for biochemical analysis. Cholesterol (>99%), pentadecanoic acid (15:0, >99%) and fatty acid methyl ester standards are obtained from Alltech/Applied Science (Deerfield, IL). Sylon BFT, 5β-cholestan-3β-ol and BCl_3/methanol (15%, w/v) are from Supelco (Bellefonte, PA). Pyridine (silylation grade) is from Pierce (Rockford, IL). Pyrogallol is from Matheson-Coleman-Bell (Cincinnati, OH). α-Tocopherol is from Sigma Chemical Co. (St. Louis, MO). Tocol was a gift from Hoffman-La Roche, Inc. (Nutley, NJ). These materials were used without further purification. H_2O is distilled in glass, deionized and charcoal-filtered. Hexanes are redistilled, reagent-grade. Ethanol is 200 proof, redistilled, USP-grade (Publicker, Linfield, PA). Methanol for liquid chromatography is high performance liquid chromatography (HPLC) grade (Fisher, Fairlawn, NJ). All solutions in organic solvents contain 50 μg/ml butylated-hydroxy-toluene (BHT).

The stock solutions of cholesterol, 5β-cholestan-3β-ol, tocol (in hexanes) and 15:0 fatty acid (in ethanol) are prepared by weighing of the material. The stock solution of α-tocopherol (in hexanes) is prepared spectrophotometrically using its molar extinction of 3170 at 292 nm (19). These stock solutions are stored tightly capped at −20 C and are stable for at least one yr.

The 15:0 internal standard solution in EtOH is dispensed with a 100 μl positive displacement SMI Micropettor (American Scientific Products, McGaw Park, IL). The standard solutions in hexane (chilled in an ice bath) are dispensed with a Pasteur pipette coupled through a section of Tygon tubing to a Gilson Pipetteman automatic pipettor. The pipettor is calibrated with a balance to deliver exactly 0.66 g of chilled hexanes (density, 20 C, hexanes = 0.66). Each pipetting method was found to have precision of better than 1% for these standard solutions.

Biochemical analysis: procedure. After collection, the sample is stored at −80 C in 1 ml of ethanol with 0.1% pyrogallol, in an 8-ml borosilicate glass vial closed with a Teflon-lined cap (#2390-H32, Arthur H. Thomas, Philadelphia, PA) and analyzed within six months of collection. These storage conditions were chosen because it was determined that α-tocopherol in plasma is stable under such conditions for at least six months.

It is difficult to directly evaluate human adipose biopsy storage conditions because duplicate samples are not completely uniform. However, the α-tocopherol/γ-tocopherol

ratio is highly reproducible even over time if subjects do not make large dietary changes (Handelman, Epstein and Dratz, unpublished data). We have observed that α-tocopherol is more sensitive to degradation than γ-tocopherol, and treatments that decompose tocopherols tend to change the α/γ ratio (unpublished results). It was found that the α-tocopherol/γ-tocopherol ratio is stable in adipose biopsy samples for at least eight months, which suggests that both tocopherols are fully stable under these conditions.

For analysis, 100 μl of 10% pyrogallol in EtOH, 100 μl of 15:0 fatty acid (10 mg/ml EtOH) and 100 μl of 30% aqueous KOH are added to the sample vial. The vial with the Teflon-lined cap is vortexed and placed in a heating block at 70 C to saponify the glycerides. At five min, the vial is taped to prevent the cap vibrating off, inverted and vortexed to bring into solution any bits of fat that might have stuck to the wall of the vial during specimen collection. Specimens then are heated at 70 C for an additional 25 min.

Vials are removed from the heating block, allowed to cool, 1.2 ml of H_2O added, followed by thorough mixing. Then 1 ml of hexane, containing both tocol (1 μg/ml) and 5β-cholestan-3β-ol (6 μg/ml) as internal standards, is added, followed by 3 ml more hexane. The vial is vortexed for 30 sec, centrifuged at 800 g for 30 sec, and the supernatant layer transferred to a clean vial. The supernatant then is split into two unequal fractions: about 1/4 for vitamin E analysis, the rest for cholesterol analysis.

For tocopherol analysis, the first supernatant aliquot is evaporated under nitrogen at 40 C, 60 μl MeOH added, and 20 μl is analyzed by reverse-phase HPLC with fluorescence detection on a Perkin-Elmer 650-LC fluorescence detector with excitation at 295 nm and emission at 340 nm (2). One hundred percent MeOH is used as mobile phase, and an Alltech C18 10μ column is employed.

For cholesterol analysis, the second supernatant aliquot is evaporated, 50 μl of a 1/1 mixture of Sylon-BFT/pyridine is added, and the sample heated at 80 C for 30 min to form the silyl derivatives. Rubber gloves are advised for steps at which contamination of samples with flakes of skin might be a problem, because skin contains relatively large amounts of cholesterol and other sterols (20), and the procedure is optimized to analyze ca. 1 μg of cholesterol or less. The sample is cooled, and the reagent evaporated under a stream of nitrogen. The sample is redissolved in 40 μl of pure 100% Sylon-BFT, and a 2 μl volume is analyzed by gas chromatography (GC) at 250 C with a flame ionization detector on a packed SE-30 column (2 m length × 2 mm i.d.) with nitrogen carrier gas at 20 cc/min flow rate. The nitrogen carrier gas used for the GC analysis must be passed through a gas-drier before the GC, otherwise residual moisture in the carrier gas will tend to hydrolyze the TMS derivatives at the high temperature on the column.

Most laboratories prepare tetramethylsilane (TMS) or other derivatives of cholesterol for GC analysis because of problems with degradation of underivatized cholesterol on the GC column (21,22). However, recent reports have employed GC analysis of underivatized cholesterol (23,24). Cholesterol is poorly soluble in Sylon-BFT alone, and a 1/1 mixture of Sylon-BFT/pyridine is used in this work for preparation of silyl derivatives. Pyridine, however, gives a very long solvent front on the gas chromatograph. Therefore, the volatile derivatization mixture is evaporated under a stream of nitrogen, and the TMS derivatives are completely dissolved in pure Sylon-BFT. The Sylon-BFT gives a very short solvent front under the gas chromatographic conditions employed.

For analysis of fatty acids, the lower layer from the saponification is acidified with HCl (4 drops of conc. HCl is sufficient to cause a strong acid reaction in the lower layer). The fatty acids then are extracted with 4 ml of hexane. A portion of the hexane extract is derivatized with BCl_3/MeOH, and the methyl esters are analyzed by GC on a SP-2340 column, with temperature programming, as described by Stone et al. (25). The percentage of each fatty acid in the specimen is obtained. Each peak is identified from its retention time by comparison with the fatty acid methyl ester standards. To determine total triglyceride in the specimen, the total fatty acid mass is multiplied by 1.05 to adjust for glycerol lost during saponification.

For quantitation of fatty acid methyl esters, a digital integrator and peak area measurements are employed. For cholesterol and tocopherol analyses, relative peak heights are utilized. The fatty acid analysis is calibrated by the 1.0 mg of 15:0 added at the start of the procedure. For calibration of tocopherol and cholesterol values, a stock solution of α-tocopherol, 700 ng/ml and cholesterol, 6.0 μg/ml is prepared in hexanes and stored at −20 C. One ml of this is dispensed at step 2, evaporated, 100 μl of 10% pyrogallol in EtOH and 1 ml of EtOH added, and the vial is carried through the analysis with the rest of the samples. This calibration is carried out in duplicate. A reagent blank also is carried through the procedure.

Because α-tocopherol elutes shortly after cholesterol on packed GC columns, the observed peak generally contains contributions from both components. However, because of partial separation, the α-tocopherol contribution to the peak height is quite small. With the GC system employed here, 0.1 μg of α-tocopherol analyzed with 1.0 μg of cholesterol increased the cholesterol peak height by only 2%, whereas errors in cholesterol estimation due to tocopherol overlap can be quite large if peak areas are used.

Tocol can be added before the saponification step, because it is not attacked by saponification if 1% pyrogallol is present. However, the 5β-cholestan-3β-ol must be added after the sample is cooled and diluted with water; otherwise, this compound is modified during the saponification to produce a split peak on the GC. Both standards were routinely added after saponification is complete. The determination of the total fatty acid content and the ratio of the 18:2/total fatty acids are not affected by saponification as compared with a direct quantitative transesterification with BCl_3/methanol.

Because the adipose is inherently nonhomogeneous, the precision of these methods was determined using aliquots of a pool of frozen human plasma and was found to be generally equal to or better than 2% for α-tocopherol and cholesterol. For the total fatty acid measurements and the percentage of major fatty acids in the specimen, 4% or better precision typically was achieved.

RESULTS

α-Tocopherol, cholesterol, triglycerides and linoleic acid in adipose tissue. Table 1 gives cholesterol/triglyceride,

TABLE 1

Biochemical Parameters of Adipose (from Buttock) from Different Subjects

Subject	Wt/wt ratios			% 18:2 in TG
	Chol/TG(× 10^3)	α-Toc/chol	α-Toc/TG(× 10^3)	
A	1.71	.242	.413	22.0
B	1.59	.139	.221	15.9
C	1.57	.197	.278	17.6
D	1.90	.314	.598	21.0
E	1.98	.341	.677	17.1
F	1.69	.148	.250	17.3
G	3.57	.144	.513	11.9
H	1.77	.220	.390	20.0
I	1.61	.212	.342	20.6

Chol, cholesterol; TG, triglyceride; α-Toc, α-tocopherol.

α-tocopherol/cholesterol, α-tocopherol/triglyceride and linoleic acid (as percentage total fatty acids) for adipose samples taken with needle biopsy from the buttock of nine adult subjects. Most specimens had a ratio of cholesterol/triglyceride in the range of 1.5–2.0 μg/mg and were concluded to be true fat. Previous reported values (mean ± SD) for various groups include 1.36 ± 0.64 μg cholesterol/mg wet wt. adipose (26), 2.08 ± 0.05 μg cholesterol/mg dry wt. adipose (27), 1.47 ± 0.23 μg cholesterol/mg adipose lipid (17), and 1.86 ± 0.53 μg cholesterol/mg triglyceride (12). Occasional subjects (subject G) were outside this range but were found to have a highly reproducible value (Fig. 2). The mean ratio of α-tocopherol/triglyceride was 402 ± 154 μg/g triglyceride, which is comparable with values reported by Kayden et al. (12). The mean wt/wt ratio of α-tocopherol to cholesterol was 0.213 ± 0.0076.

Linoleic acid. As predicted from the results of Hirsch et al. (1) and others (4,28), the percentage of linoleic acid in specimens from each subject showed little or no change between clinic visits over the time frame of this study (several weeks to months). The 18:2 content is expressed in Table 1 as percentage of the six major fatty acids in human adipose, 14:0, 16:0, 16:1, 18:0, 18:1 and 18:2. For most specimens, these are greater than 95% of the total. The mean linoleic acid from subjects in this study was 18.2% of total fatty acids.

Cholesterol/triglyceride ratios. The punch biopsy and needle biopsy samples described above were evaluated for their cholesterol and triglyceride content, and it was found that only the needle biopsy procedure reliably provided specimens with lipid characteristics appropriate for adipose tissue. Cholesterol/triglyceride ratios in samples collected by punch biopsy are compared with samples collected by the modified needle biopsy in Figure 2. Although all the punch biopsy samples appeared to consist of pure adipocytes on microscopic examination, Figure 2 shows that 20% of the samples collected by punch biopsy had a very aberrant ratio of cholesterol/triglyceride, indicating the presence of non-adipose tissue. Even the punch biopsy samples with the lower, more normal values of cholesterol/triglyceride ratios showed rather large scatter in this ratio.

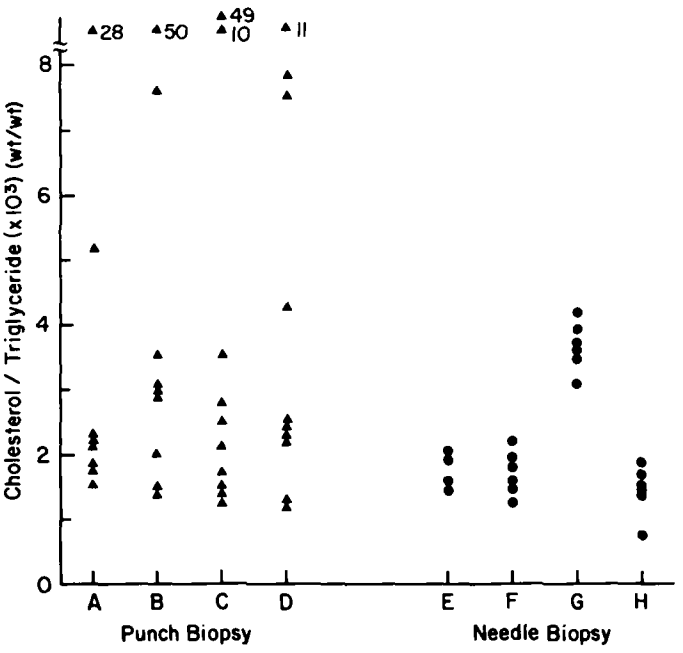

FIG. 2. Cholesterol/triglyceride ratios for human adipose samples collected from the buttock by punch biopsy (▲) and by needle biopsy (●), with multiple sampling from the same subjects. The different subjects are denoted by capital letter codes.

Extreme values of the cholesterol/triglyceride ratio are not observed with the modified needle biopsy method, and the values for each subject cluster more tightly compared with the punch method, as shown in Figure 2. Subject H had one low and unexplained value of cholesterol/triglyceride. All the values for subject G are reproducibly in the range of three to four parts cholesterol/1000 parts triglyceride, and we believe this high value is the normal state for this subject.

Comparison of reproducibility of the punch biopsy and needle biopsy methods. The key parameters evaluated in this investigation are the ratios of α-tocopherol to the two different denominators available, cholesterol and triglyceride. A reliable method would obtain reproducible results for the ratio α-tocopherol/denominator when sampling an individual subject at the same site on the same day. The reproducibility of the punch and needle biopsy methods for each denominator were evaluated from an analysis of a substantial number of within-day replicate samples collected from a number of subjects (data not shown). For 14 sets of replicates collected with the punch biopsy, the mean within-day difference for the α-tocopherol/cholesterol ratio was 44.6% and for the α-tocopherol/triglyceride ratio, the mean difference was 26.0%. For 10 sets of replicates collected with the needle biopsy, the mean difference in α-tocopherol/cholesterol ratio was 8.8%, which was the most reproducible parameter found in replicate sampling of the same subject, and the mean difference in α-tocopherol/triglyceride ratio was 15.8%.

Variation in α-tocopherol/cholesterol and α-tocopherol/triglyceride ratio on replicate sampling at different sites. The development of the needle biopsy method provided reproducible values of the ratio of tocopherol/denominator

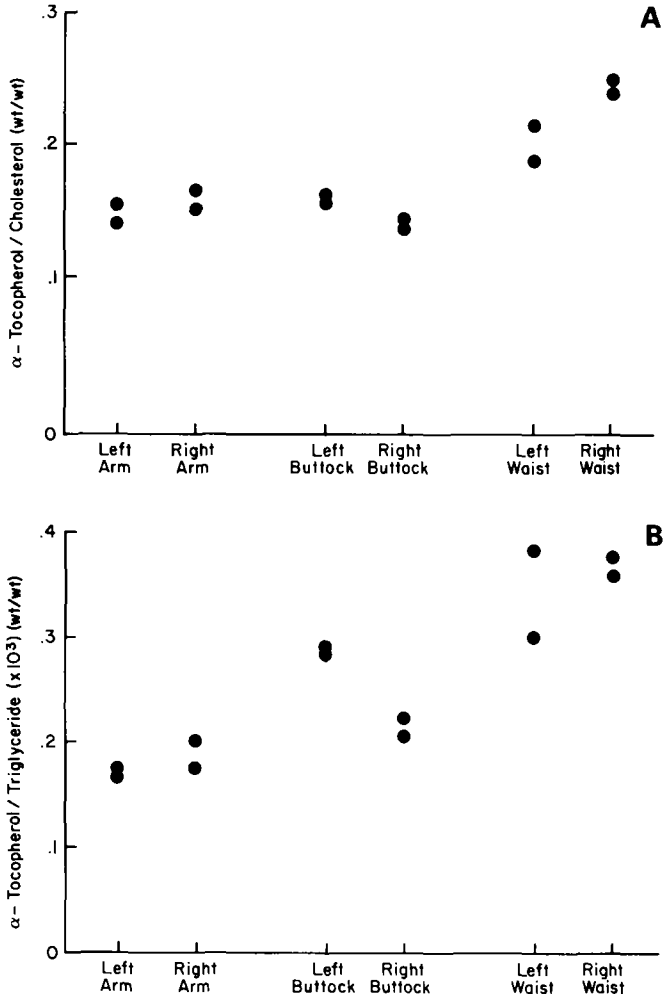

FIG. 3. (A) Human adipose α-tocopherol/cholesterol ratios determined by duplicate sampling with needle biopsy of one subject on same day at different anatomical sites. (B) Human adipose α-tocopherol/triglyceride ratios determined by duplicate sampling with needle biopsy of same subject as in A on the same day at different anatomical sites.

for within-day replicate sampling of individual subjects, using the cholesterol denominator. Once this was established, we evaluated the anatomical variation of the α-tocopherol/lipid ratios at different sites on the same subject. One subject was sampled at six sites, in duplicate on right and left upper arm, right and left buttock, and right and left waist, using the needle biopsy. Figure 3A shows the α-tocopherol/cholesterol ratios for this subject at each site, and Figure 3B shows the α-tocopherol/triglyceride ratios. The reproducibility of duplicate samples shows that a reliable biopsy procedure has been established. In this subject, the waist fat has substantially more α-tocopherol (per weight cholesterol or per weight triglyceride) than the other sites.

DISCUSSION

The methods described were developed with the primary goal of following the uptake and turnover of α-tocopherol in human adipose tissue using deuterated tocopherol in future experiments. This information is required to evaluate the use of adipose tocopherol for assessment of the average long-term vitamin E status in humans. Tissue was taken from subjects as often as twice a month, with two (or more) samples taken per subject per clinic visit. The Hirsch procedure (1) was found to be time-consuming and fatiguing to use for the physician. It led to an effective limit of five to eight samples per half-day session, and furthermore, it often failed to yield adequate amounts of tissue for the work described here. The conventional biopsy punch, which takes a 2-mm core, was unacceptable to the subjects when used frequently because it leaves small scars. The punch biopsy method (16) was investigated because it is a simple and facile procedure, and many dermatologists have experience with it; however, the unreliability of using punch biopsies for biochemical adipose sampling should be emphasized. With the punch method, specimens that have a lipid profile not at all characteristic of adipose often are obtained. Fat collected from very near the dermis may be contaminated with skin components, which are known to be high in cholesterol (20), and this caused a large variation in the α-tocopherol/cholesterol ratio.

The method pioneered by Beynen and Katan (14), which combines needle biopsy and a Vacutainer to create suction, proved very satisfactory with simple modifications. The modified needle biopsy method has entailed no objections from the subjects, especially because it leaves no scar. Specimens collected with the needle biopsy consistently have been biochemically normal for adipose as classically described from adipose obtained by surgical means. The needle biopsy method provides quite ample amounts of tissue (2–10 mg, in most cases) for analysis by the microchemical assays described in Experimental. This method is now a simple and routine procedure in our ongoing investigations of human adipose tissue.

There are several modifications to the method of Kayden et al. (12) for biochemical analysis of α-tocopherol and other components in microbiopsies. This method employed tocol as internal standard, and no internal standard was used before (12). This method uses internal standard GC for cholesterol, and fatty acid analysis carried out simultaneously on the same small specimens. We used pyrogallol to protect α-tocopherol during saponification, whereas ascorbic acid (12) was not reliable for this procedure in our hands. However, it has been noted that some lots of pyrogallol may not yield reliable results (29), so each batch of pyrogallol should be checked to ensure that it protects vitamin E during saponification.

The cholesterol/triglyceride ratio on each specimen is useful because it validates that a true adipose sample has been obtained. We hypothesize that both cholesterol and triglyceride levels in adipose are relatively stable over time in subjects who are not gaining or losing weight and that each may be useful as a denominator to express tissue levels of other adipose constituents. This hypothesis is being explored in more studies. The ratio α-tocopherol/cholesterol appears most promising in long-term studies of α-tocopherol turnover in adipose. Our laboratories are carrying out studies of long-term turnover of α-tocopherol and other tocopherols in adipose and are evaluating the effects of dietary supplementation on these parameters.

We used a food-frequency questionnaire (18) to evaluate the lipid nutritional status of most of our subjects. The

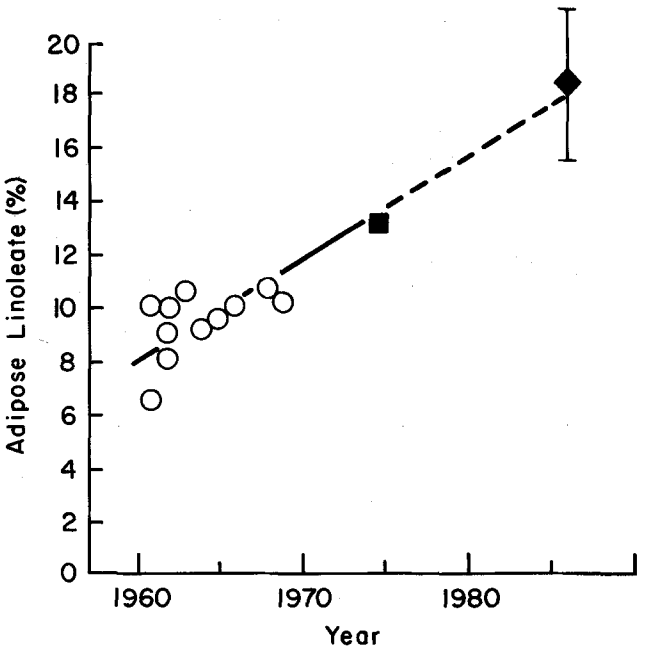

FIG. 4. Human adipose linoleate as percentage of major fatty acids reported from 1961 to 1975, compared with the average value determined in this study. (○), Data summarized by Insull and Bartsch (30); (■), data from Witting and Lee (31); (◆), this study.

food frequency questionnaires were scored by W. C. Willett's laboratory at Harvard University. The diet analysis yielded several parameters that are suitable for identifying individuals who modify their lipid nutrition away from the norm: percentage calories as fat, percentage total dietary fat as linoleic acid, and cholesterol/Kcal diet. Health-conscious individuals would be expected to have a lower cholesterol/Kcal, lower percentage calories as fat and higher percentage total fat as 18:2. This study group had essentially the same percentage calories as fat and percentage total dietary fat as linoleic acid as the Willett normal population. This study group had slightly higher cholesterol/Kcal diet than the Willett population. Therefore, it may be concluded that the subjects in this study showed no significant tendency to reduce fat intake or to increase percentage linoleic acid compared with a control population.

The findings in this study with percentage 18:2 in adipose, taken with earlier data, suggest that important demographic dietary trends may be occurring in American populations. Insull and Bartsch (30) summarized adipose fatty acid data from 11 studies of Americans carried out between 1960 and 1967. The average 18:2 was 9.5% of the major fatty acids. Witting and Lee (31) in 1974 found that 18:2 was 13% of major fatty acids in adipose. In the present study group, 18:2 was 18.2% of major fatty acids. Witting and Lee pointed out a striking increase in adipose 18:2 from the early 1960s to 1974. The same trend has been noted in the U.S. for the years 1958–1975 purely from literature data (32). The data from this study fits smoothly on with this trend, as shown in Figure 4, and extends the data for 10 years. This finding suggests a substantial, continuing increase in 18:2 in the American diet. This trend is consistent with a reported increase in vegetable fat consumption in the U.S. from 25% of total dietary fat in 1947–1949 to 42% of total dietary fat in 1980 (33). Such an increase may be significant, because 18:2 has been suggested as a risk factor for increased peroxidative damage to tissues (34). This suggests that a larger group of Americans should be sampled periodically, so that long-term trends in adipose fatty acids can be followed in a larger population.

Adipose collected from the waist appeared different in composition from adipose collected from arm and buttock (Fig. 4), although this point was not investigated in detail. Klein et al. (35), using the marker methyl-tetradecanoic acid to compare fatty acid turnover rates at these three sites, found that turnover was more rapid at the waist. Comparison of the ratios of α-tocopherol to cholesterol and to triglyceride at anatomical sites other than the buttock may warrant further investigation.

The biopsy method as described may have broader applications. The method might be suitable for evaluating the change in the ratio of cholesterol/triglyceride in adipose over time, which is of interest in studying the effects of certain weight-loss protocols (17). It may be suitable for the study of the metabolism and turnover of other fat-soluble vitamins, such as retinol and carotenoids, and for the study of fat-soluble drugs, exogenous fat-soluble compounds, such as environmental pollutants (7,8), and certain hormones, such as pregnancy and lactation hormones. As discussed by Erickson (36), the protocol described may be applicable to the analysis of PCBs in adipose tissue. The adipose is a useful indicator storage site for toxic lipid peroxides (34,37), which might be correlated with disease states (38) using new, highly sensitive and specific assay procedures for lipid peroxides (39,40).

ACKNOWLEDGMENTS

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REFERENCES

1. Hirsch, J., Farquhar, J.W., Ahrens, E.H. Jr., Peterson, M.L., and Stoffel, W. (1960) *Am. J. Clin. Nutr. 8,* 499–511.
2. Handelman, G.J., Machlin, L.J., Fitch, K., Weiter, J.J., and Dratz, E.A. (1985) *J. Nutr. 115,* 807–813.
3. Baker, H., Handelman, G.J., Short, S., Machlin, L.J., Bhagavan, H.N., Dratz, E.A., and Frank, O. (1986) *Am. J. Clin. Nutr. 43,* 382–387.
4. Dayton, S., Hashimoto, S., Dixon, W., and Pearce, M.L. (1966) *J. Lipid Res. 7,* 103–111.
5. Bieri, J.G. (1972) *Ann. NY Acad. Sci. 203,* 181–191.
6. Machlin, L.J., Keating, J., Nelson, J., Brin, M., Filipski, R., and Miller, O.N. (1979) *J. Nutr. 109,* 105–109.
7. Morgan, D.P., and Roan, C.C. (1971) *Arch. Env. Health 22,* 301–308.
8. Wolff, M.S., Anderson, H.A., Rosenman, K.D., and Selikoff, I.J. (1979) *Bull. Env. Contamin. Toxicol. 21,* 775–781.
9. McMasters, V., Lewis, J.K., Kinsell, L.W., Van der Veen, J., and Olcott, H.S. (1965) *Am. J. Clin. Nutr. 17,* 357–359.
10. McMasters, V., Howard, T., Kinsell, L.W., Van der Veen, J., and Olcott, H.S. (1867) *Am. J. Clin. Nutr. 19,* 622–626.
11. Bieri, J.G., and Poukka Evarts, R. (1975) *Am. J. Clin. Nutr. 28,* 717–720.
12. Kayden, H.J., Hatam, L.J., and Traber, M.G. (1983) *J. Lipid Res. 24,* 652–666.
13. Kayden, H.J. (1983) in *Biology of Vtamin E* (Porter, R., and Whelan, J., eds.) pp. 70–91, Pitman, London.
14. Beynen, A.C., and Katan, M.B. (1985) *Am. J. Clin. Nutr. 42,* 317–322.

15. Christakis, G. (1965) *J. Lipid Res. 6,* 427-428.
16. Robinson, J.K. (1986) *Fundamentals of Skin Biopsy,* pp. 19-24, Yearbook Medical Publishers, Chicago, IL.
17. Angel, A., and Bray, G.A. (1979) *Eur. J. Clin. Invest. 9,* 355-362.
18. Willett, W.C., Reynolds, R.D., Cottrell-Hoehner, M.S., Sampson, L., and Browne, M.L. (1987) *J. Am. Diet. Assoc. 87,* 43-47.
19. Baxter, J.G., Robeson, C.D., Taylor, J.D., and Lehman, R.W. (1943) *J. Am. Chem. Soc. 65,* 918-924.
20. Myant, N.B. (1981) *The Biology of Cholesterol and Related Steroids,* pp. 130-131, William Heinemann, London.
21. Horning, E.C., Maddock, K.C., Anthony, K.V., and Vandenheuvel, W.J.A. (1963) *Anal. Chem. 35,* 526-532.
22. Fumagalli, R. (1976) in *Lipid Chromatographic Analysis,* 2nd edn. (Marinetti, G.V., ed.) Vol. 3, pp. 791-817, Marcel Dekker, New York.
23. Brunnekreeft, J.W.I., Boerma, G.J.M., and Leijnse, B. (1983) *Ann. Clin. Biochem. 20,* 360-363.
24. Traber, M.G., Kayden, H.J., Green, J.B., and Green, M.H. (1986) *Am. J. Clin. Nutr. 44,* 914-923.
25. Stone, W.L., Farnsworth, C.C., and Dratz, E.A. (1979) *Exp. Eye Res. 28,* 387-397.
26. Khan, B., Cox, G.E., and Asdel, K. (1963) *Arch. Pathol. 76,* 369-381.
27. Crouse, J.R., Grundy, S.M., and Ahrens, E.H. Jr. (1972) *J. Clin. Invest. 51,* 1292-1296.
28. Fleischman, A.I., Hayton, T., Bierenbaum, M.L., and Watson, P. (1968) *Lipids 3,* 147-150.
29. Hatam, L.J., and Kayden, H.J. (1979) *J. Lipid Res. 20,* 639-645.
30. Insull, W., and Bartsch, G.E. (1967) *Am. J. Clin. Nutr. 20,* 13-23.
31. Witting, L.A., and Lee, L. (1975) *Am. J. Clin. Nutr. 28,* 577-583.
32. Katan, M.B., and Beynen, A.C. (1981) *Lancet 2,* 371.
33. Welsh, S.O., and Marston, R.M. (1982) *J. Am. Diet. Assoc. 81,* 120-125.
34. van Kuijk, F.J.G.M., Thomas, D.W., Stephens, R.J., and Dratz, E.A., in *Biological Effects of Lipid Peroxidation* (Sevanian, A., ed.) Academic Press, New York, in press.
35. Klein, R.A., Halliday, D., and Pittet, P.G. (1980) *Lipids 15,* 572-579.
36. Erickson, M.D. (1985) *Analytical Chemistry of PCB's,* p. 167, Butterworths, London.
37. Glavind, J., Nannestad Hansen, F., and Sylven, C. (1976) *Int. J. Vit. Nutr. Res. 46,* 258-261.
38. Handelman, G.J., and Dratz, E.A. (1986) *Adv. Free Radicals Biol. Med. 2,* 1-89.
39. van Kuijk, F.J.G.M., Thomas, D.W., Stephens, R.J., and Dratz, E.A. (1985) *J. Free Radicals Biol. Med. 1,* 215-225.
40. van Kuijk, F.J.G.M., Thomas, D.W., Stephens, R.J., and Dratz, E.A. (1985) *J. Free Radicals Biol. Med. 1,* 387-393.

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Use of a Fluorescent Radiolabeled Triacylglycerol as a Substrate for Lipoprotein Lipase and Hepatic Triglyceride Lipase

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A fluorescent radiolabeled triacylglycerol has been synthesized by using a fluorescent fatty acid (pyrene decanoic acid) and a radiolabeled oleic acid. This analog of the natural substrate, 1(3)pyrene decanoic-2,3 (1,2)-dioleoyl-*sn*-glycerol, has been tested as substrate for determining lipoprotein lipase and hepatic triacylglycerol lipase activities in post-heparin plasma. Optimal conditions for the determination of the two post-heparin plasma lipases were similar to those using radiolabeled triolein. Using this substrate, both post-heparin lipases exhibited their characteristic properties (pH optimum and effect of inhibitors) and attacked external ester bonds (1 or 3) containing pyrene decanoic and oleic acids at a similar rate. *Lipids 23,* 605–608 (1988).

Two kinds of lipase, lipoprotein lipase (EC 3.1.1.34) and hepatic triacylglycerol lipase (EC 3.1.1.3), are released into plasma when heparin is intravenously administered. Both enzymes play important roles in the metabolism of lipoproteins: LPL in the degradation of triglycerides of triglyceride-rich lipoproteins, such as chylomicrons and VLDL, and H-TGL in the hydrolysis of triacylglycerols of intermediate-sized particles (1). These lipolytic enzymes exhibited the same substrate specificity (against long-chain triacylglycerols) and also a similar stereospecificity on external (1 or 3) acyl ester bonds of triacylglycerol (2–5). Thus, as emphasized by Baginsky (6) the direct determination of each activity needs the use of either effectors or antibodies or separation on a heparin-sepharose column (but this latter method is not useful for routine determinations) (7–10).

Post-heparin lipolytic activity commonly is measured with radioisotopically labeled substrate (11). Colorimetric techniques involving the determination of free fatty acids have been reported (12). Another assay described the use of a dansyl fluorescent lipid to follow the reaction of lipoprotein lipase (13). Finally, a recent study used 1,2-dipalmitoyl-3-β-2-furylacryloyltriacylglycerol as a chromophoric substrate (14).

In this paper, we report a new fluorometric method for determining lipoprotein lipase and hepatic triacylglycerol lipase in post-heparin plasma. Fluorometric assays of lipases such as pancreatic lipase (15) and lysosomal acid lipase (16) have been used successfully. We have evaluated this sensitive and specific method for determining post-heparin plasma activities and compared the results with those of radioactive method.

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Abbreviations: LPL, lipoprotein lipase; H-TGL, hepatic triacylglycerol lipase; $P_{10}OOG$, 1(3)pyrene decanoyl-2,3(1,2)-dioleoyl-*sn*-glycerol; VLDL, very low density lipoproteins.

MATERIALS AND METHODS

Chemicals. Tri[9,10-^{3}H]oleoylglycerol was obtained from Amersham (Les Ulis, France), [9,10-^{3}H]oleic acid was from NEN (Dreieich, FRG), 10-(1-pyrene)-decanoic acid, triolein, lysophosphatidylcholine and albumin free fatty acids were obtained from Sigma Chemical Co. (St. Louis, MO). Heparin (5,000 U/ml) was purchased from Roche (Paris, France).

We synthesized the fluorescent radiolabeled substrate 1(3)-pyrenedecanoyl-2,3(1,2)-di[9,10-^{3}H]oleoylglycerol ($P_{10}OOG$) using a three-step method including a process of esterification of (R)-(+)-2,2-dimethyl 1,3-dioxolane-4-methanol by 25 μmol of pyrene decanoic acid (17) followed by the hydrolysis of the ketal function (18) and the esterification of the obtained monoglyceride by 50 μmol of [9,10-^{3}H]oleic acid (17). The product was purified by thin layer chromatography using a solvent system of light petroleum/diethyl ether (80:20, v/v); the purity and the structure of the compound were tested by gas chromatography (after hydrolysis and methylation) and by proton nuclear magnetic resonance spectroscopy.

Plasma samples. Post-heparin plasma was prepared from rabbit blood drawn 10 min after IV administration of 175 IU heparin per kg body weight. Plasma was kept frozen (−20 C) until use.

Enzyme assays. We have used the specific assays of lipoprotein lipase and hepatic lipase activities described by Nilsson-Ehle and Ekman (19). These authors employed a combination of the differential catalytic properties of the lipases to selectively measure one in the presence of the other. Under these optimal conditions, we compared our fluorescent radiolabeled triacyglycerol with the radiolabeled triolein (used as reference method).

Assay for hepatic triacylglycerol lipase (H-TGL). Labeled triacylglycerol (35 nmol) and unlabeled triolein (7.92 μmol) were mixed with 0.6 μmol lysophosphatidylcholine. After removal of solvents under a stream of nitrogen, 1.8 ml of 0.2 M Tris-HCl buffer (pH 9.0) and 1.2 ml 1% (w/v) bovine serum albumin in the same buffer were added. The mixture was sonicated with a Branson sonifier in 30-sec intervals for a total of three min and used as a substrate. The standard H-TGL assay contained 0.1 ml buffered substrate and the enzyme solution (post-heparin plasma adjusted to 1 M NaCl by adding an equal volume of 2 M NaCl) in a final volume of 0.2 ml. After incubation (30 min at 37 C), the liberated fatty acids were selectively extracted using the method of Belfrage and Vaughan (20). After mixing and centrifuging, we measured the liberated fatty acid extracted in the upper aqueous phase radiometrically (19) and fluorometrically with a Jobin-Yvon JY 3C spectro fluorometer (excitation 342 nm, emission 398 nm) calibrated by using a standard of P_{10} fatty acid prepared in the assay conditions.

Assay for lipoprotein lipase (LPL). The substrate was prepared by sonicating a mixture of 35 nmol labeled triacylglycerol, 7.92 μmol unlabeled triolein, 0.6 μmol

lysophosphatidylcholine and 2.4 ml 0.2 M Tris-HCl (pH 8.0) buffer. After sonication, 0.3 ml plasma as source of apo CII but devoid of lipase activity and 0.3 ml 4% (w/v) bovine serum albumin in 0.2 M Tris-HCl (pH 8.0) buffer were added and the substrate shaken on a vortex mixer for 15 sec. The standard assay contained 0.1 ml substrate, 0.075 M NaCl and enzyme in a final volume of 0.2 ml. After incubating for 30 min at 37 C, the liberated fatty acids were extracted and determined as indicated for H-TGL assay.

Test of inhibitors. To discriminate between LPL and H-TGL activities in the whole post-heparin plasma, we have utilized the classical inhibitors of LPL (1 M NaCl) and of H-TGL (50 mM SDS). These inhibitors were preincubated with the post-heparin plasma in the optimal conditions defined by Nilsson-Ehle and Ekman (19) and Baginsky (6) (experimental details are in Table 1).

Enzymatic activities by the radioactive method were calculated as described by Nilsson-Ehle and Schotz (11). Lipase activities by the fluorometric method were measured using a standard of P_{10} fatty acid. One mU of enzyme activity was defined as the amount of the enzyme in 1 ml of enzyme preparation, which released 1 nmol fatty acid per min at 37 C.

RESULTS

$P_{10}OOG$, the synthetic fluorescent analog of natural triacylglycerol, is hydrolyzed by LPL and H-TGL. Therefore, it can be used as a substrate for determining the lipase activities in post-heparin plasma.

Enzyme activity was directly proportional to the amount of post-heparin plasma up to 10 µl for both enzymes (Fig. 1). In the standard assay, we generally used 10 µl of plasma as enzyme source.

Kinetic curves of substrate hydrolysis vs time were linear for at least 60 min at 37 C (Fig. 2).

These enzymes showed similar optimum pH and activity with either the fluorescent $P_{10}OOG$ or the radiolabeled triolein as substrate (Fig. 3). The pH profile of the hydrolysis of both substrates showed one peak of lipolytic activity with an optimum at pH 9.0 for H-TGL (Fig. 3A). LPL assay had a pH optimum of about 8.0 using either substrate (Fig. 3B).

The sodium dodecyl sulfate and sodium chloride were controlled to affect the activity of H-TGL and LPL differently. Lipase activity of post-heparin plasma measured

TABLE 1

Effect of NaCl and Sodium Dodecyl Sulfate on Lipase Activities in Post-Heparin Plasma

Treatment of plasma	Enzymatic activity (% of control)	
	H-TGL assay	LPL assay
Control	100	100
NaCl, 10 min at 26 C[a]	100	25
NaCl, 10 min at 37 C[a]	110	15
SDS, 60 min at 26 C[b]	6.9	61
SDS, 60 min at 26 C and 1 M NaCl	—	10

[a]Duplicate samples of post-heparin plasma (100 µl) were diluted to 1 ml with 0.15 M NaCl. To 100 µl of diluted plasma 100 µl 2 M NaCl was added according to Nilsson-Ehle and Ekman (19).

[b]Post-heparin plasma was preincubated with 50 mM sodium dodecyl sulfate in 0.2 M Tris-HCl buffer, pH 8.2 during 60 min at 26 C according to Baginsky (6).

Experiments were done in duplicate.

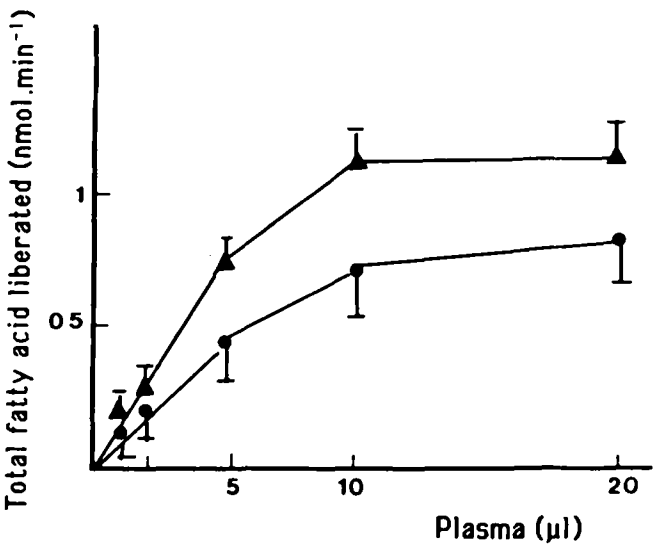

FIG. 1. Hydrolysis of $P_{10}OOG$ by hepatic triacylglycerol lipase (▲) and lipoprotein lipase (●) when increasing amounts of enzyme source (post-heparin plasma) in the standard assay conditions described in Material and Methods. Average and range (bars) of five experiments.

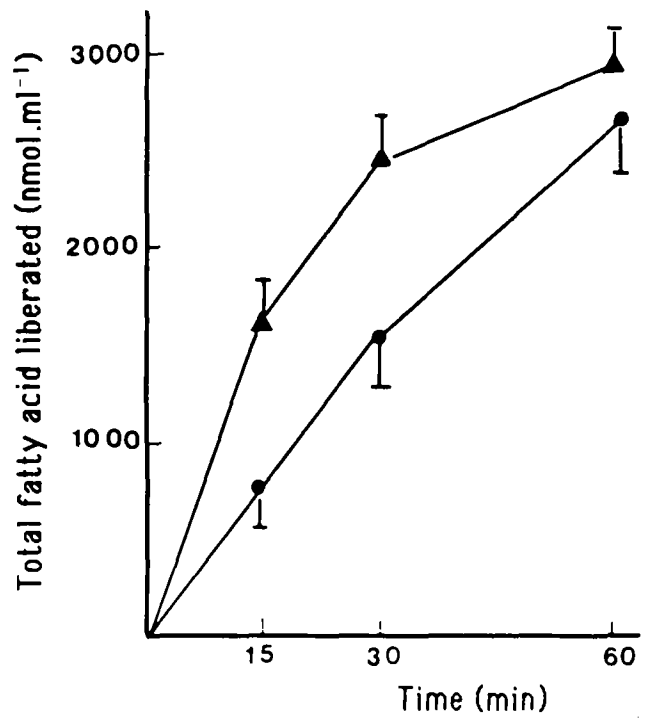

FIG. 2. Hydrolysis of $P_{10}OOG$ by hepatic triacylglycerol lipase (▲) and lipoprotein lipase (●) as a function of time. Enzyme activities were assayed under standard assay conditions reported in Material and Methods. Average and range (bars) of five experiments.

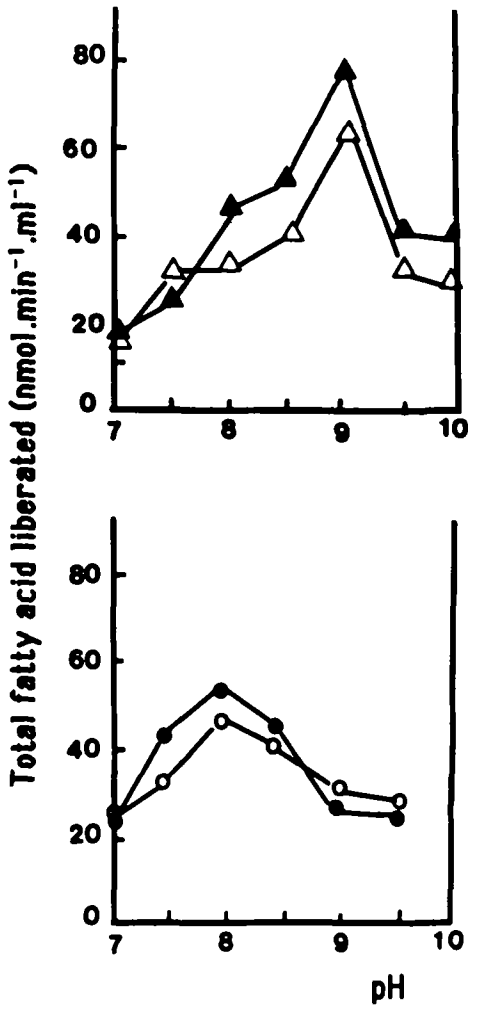

FIG. 3. Effect of pH on hepatic triacylglycerol lipase (upper) and lipoprotein lipase (lower) activities. Enzyme assays were performed as indicated for the standard assays but using various 0.5 M Tris-HCl buffers (pH varying between 7 and 10). Using fluorescent $P_{10}OOG$ (filled symbols), and radiolabeled triolein (open symbols), the enzyme activities were expressed as total fatty acid liberated (nmol/min/ml).

with LPL assay showed an almost complete inhibition by addition of 1 M NaCl (final concentration) to the assay (Table 1), a well-known characteristic of LPL activity. On the contrary, the lipase activity measured with H-TGL assay was unaffected by NaCl (Table 1). On the other hand, preincubation of post-heparin plasma with 50 mM sodium dodecyl sulfate during 60 min at 26 C suppressed the H-TGL activity (Table 1). However, we also have noted that inhibition of LPL activity can occur. As a control that the activity remaining after SDS treatment is really LPL, we have tested the inhibition of this enzyme activity by 1 M NaCl. As expected, this activity was almost completely inhibited, therefore demonstrating that it was only due to LPL.

We have compared in the synthetic substrate the liberation rate of pyrene decanoic and oleic acids as a function of time (Table 2). The results showed that the time course of substrate hydrolysis by LPL and H-TGL is the same regardless of the fluorescent or the radioactive moiety considered.

TABLE 2

Hydrolysis of Synthetic Substrate: Comparison of the Liberation Rate of Pyrene Decanoic and Oleic Acids

	Fluorescence		Radioactivity	
Time	LPL	H-TGL	LPL	H-TGL
15 min	702	1701	605	1876
30 min	1549	2535	1459	2285
60 min	2640	2956	2562	3026

The data are expressed as nmol total fatty acid released by 1 ml plasma. Experiments were performed in triplicate.

DISCUSSION

This paper demonstrates that the two post-heparin lipases that split the ester linkages of natural substrates (11) also hydrolyze the synthetic fluorescent analog of the natural substrate ($P_{10}OOG$). Using this substrate, pH profiles were quite similar to those observed using trioleoylglycerol as substrate (Fig. 3); these results were quite consistent with the pH optima reported in the literature (19). Lipoprotein lipase rapidly hydrolyzes synthetic substrate prepared as described by Nilsson-Ehle and Ekman (19). Specific measurements of this lipase activity can be performed at pH 8.0, at 0.15 M NaCl and in the presence of plasma, adding apolipoprotein CII as activator (21). Hepatic triacylglycerol lipase activity can be measured selectively using the synthetic substrate in 0.2 M Tris-HCl (pH 9.0) containing 1 M NaCl.

H-TGL differs in a number of aspects from the lipoprotein lipase. In fact, sodium chloride and sodium dodecyl sulfate affect the activity of H-TGL and LPL differently. H-TGL is not inhibited by NaCl and not stimulated by apolipoprotein CII. These data are in good agreement with those reported in literature (19,22,23) with natural substrate. H-TGL activity is suppressed by preincubation with SDS, in agreement with the results of Baginsky and Brown (8). We also noted that LPL activity is decreased by preincubation of plasma with SDS. Elsewhere, it is noteworthy that SDS also partially inhibits the activity measured by LPL assay (39%). This is quite consistent with the data reported by Baginsky (6), who suggests that this apparent inhibition could be due to the inactivation of H-TGL, which interferes in LPL assay. However, in our assay conditions, a partial inhibition of LPL by SDS cannot be absolutely excluded.

These results concerning the liberation rate of pyrene decanoic and oleic acids as a function of time demonstrate that ester linkage containing P_{10}-fatty acid is hydrolyzed similarly to that containing the radioactive oleic acid. These results suggest that only external (i.e. 1 or 3) ester bonds are attacked by both lipases in agreement with previous reports (2-5). It also is noteworthy that the presence of pyrene nucleus does not hinder the interaction of the synthetic $P_{10}OOG$ with the enzymatic sites of both lipases, as demonstrated by the similar liberation rates of P_{10} and oleic acid from the doubly labeled substrate (Table 2).

Finally, we emphasize that the assay utilizing the fluorescent radiolabeled $P_{10}OOG$ exhibits a good sensitivity

(limit of detection was around 1 nmol/min/ml in the used conditions), much better than that of the assays utilizing chromophoric substrates (24). Thus, the fluorescent substrates can be used diluted many fold (300-fold in our assay) with the natural substrate, thereby considerably reducing the amount of the substrate analogue used in the reaction mixture.

In conclusion, the use of synthetic fluorescent triacylglycerol in the presence of natural triacylglycerol provides an easy, convenient assay system for estimating hepatic lipase and lipoprotein lipase activities.

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REFERENCES

1. Rubinstein, A., Gibson, J.C., Paterniti, J.R., Kakis, G., Little, A., Ginsberg, H.N., and Brown, W.V. (1985) *J. Clin. Invest. 75,* 710–721.
2. Somerharju, P., Kuusi, T., Paltauf, F., and Kinnunen, P.K.J. (1978) *FEBS Lett. 96,* 170–172.
3. Paltauf, F., and Wagner, E. (1976) *Biochim. Biophys. Acta 431,* 359–362.
4. Akesson, B., Gronowitz, S., and Herslôf, B. (1976) *FEBS Lett. 71,* 241–244.
5. Paltauf, F., Esfandi, F., and Holasek, A. (1974) *FEBS Lett. 40,* 119–123.
6. Baginsky, M.L. (1981) in *Methods in Enzymology* (Lowenstein, J.M., ed.), Vol. 72, pp. 325–338, Academic Press, New York.
7. Krauss, R.M., Windmueller, H.G., Levy, R.I., and Fredrickson, D.S. (1973) *J. Lipid Res. 14,* 286–295.
8. Baginsky, M.L., and Brown, W.V. (1979) *J. Lipid Res. 20,* 548–556.
9. Ehnholm, C., Shaw, W., Greten, H., and Brown, W.V. (1975) *J. Biol. Chem. 250,* 6756–6761.
10. Boberg, J., Augustin, J., Baginsky, M.L., Teheda, P., and Brown, W.V. (1977) *J. Lipid Res. 18,* 544–547.
11. Nilsson-Ehle, P., and Schotz, M.C. (1976) *J. Lipid Res. 17,* 536–541.
12. Fruchart, J.C., Dewailly, P., Jaillard, J., and Sezille, G. (1974) *Ann. Biol. Clin. 32,* 237–240.
13. Johnson, J.D., Taskinen, M.R., Matsuoka, N., and Jackson, R.L. (1980) *J. Biol. Chem. 255,* 3466–3470.
14. McFarland, J.T., and Rojas, C. (1986) *Biochim. Biophys. Acta 876,* 438–449.
15. Salvayre, R., Nègre, A., Radom, J., and Douste-Blazy, L. (1986) *Clin. Chem. 32,* 1532–1536.
16. Nègre, A., Salvayre, R.S., Dagan, A., and Gatt, S. (1985) *Clin. Chim. Acta 149,* 81–88.
17. Neises, B., and Steglich, W. (1978) *Angew. Chem. Int. Ed. Engl. 17,* 522–523.
18. Jensen, R.G., and Pitas, R.E. (1976) *Adv. Lipid Res. 14,* 213–247.
19. Nilsson-Ehle, P., and Ekman, R. (1977) *Artery 3,* 194–209.
20. Belfrage, P., and Vaughan, M. (1969) *J. Lipid Res. 10,* 341–344.
21. Nilsson-Ehle, P., Garfinkel, A.S., and Schotz, M.C. (1980) *Ann. Rev. Biochem. 49,* 667–693.
22. Ehnolm, C., Shaw, W., Greten, H., and Brown, W.V. (1975) *J. Biol. Chem. 250,* 6757–6761.
23. Kinnunen, P.K.J., and Ehnholm, C. (1976) *FEBS Lett. 65,* 354–357.
24. Nègre, A., Salvayre, R., Dagan, A., Borrone, C., and Gatt, S. (1985) *Anal. Biochem. 145,* 398–405.

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A Simplified Approach to the Analysis of Subclasses of Phospholipids: Application to Human Platelets

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A procedure for the determination of the proportions of diacyl, alkenylacyl and alkylacyl subclasses of glycerophospholipids was developed. The procedure involves: (1) acid methanolysis of the phospholipid followed by Bligh/Dyer extraction of fatty acid methyl esters (FAME) derived from acyl chain types, dimethylacetals (DMA) derived from alkenyl ether chain types, and lysoalkyl phosphatidic acids (lysoalkyl-PA) derived from alkyl ether chain types; and (2) subsequent acetolysis to convert the lysoalkyl-PA to monoalkyl glycerol diacetates (MAGD). GLC analysis and quantitation (using internal standard, 21:0 FAME) of FAME, DMA and MAGD allowed calculation of the proportions of the three molecular subclasses. The methanolysis/acetolysis procedure gave an overall mean phospholipid recovery of 95 ± 3%. Analysis of the major phospholipids in four separate preparations of fresh resting human platelets by this procedure showed the following range of molecular subclasses: phosphatidylcholine (PC), 86–92 mol % diacyl, 6–10 mol % alkylacyl and 2–3 mol % alkenylacyl; and phosphatidylethanoline (PE), 39–60 mol % diacyl, 5–8 mol % alkylacyl and 34–55 mol % alkenylacyl. The results of these subclass analyses were in general agreement with those reported in the literature.
***Lipids 23,* 609–614 (1988).**

Platelet phospholipids consist of complex mixtures of molecular species of diacyl, alk-1-enyl, and alkylacyl subclasses; the relative proportions of these subclasses vary with the phospholipid component and the animal species involved and differ from other blood cell types (1–4).

Conventional methods of analysis of phospholipid subclasses employ cleavage of alk-1-enyl groups by exposure to HCl fumes (2–4) or treatment with mercuric chloride solution (5,6), followed by thin layer chromatography (TLC) separation of the aldehyde and lysophospholipid products and the unreacted diacyl and alkylacyl phospholipids; quantitation of the lyso-PL by P-analysis gives the alk-1-enyl class content. Phospholipase C hydrolysis of the unreacted diacyl and alkylacyl phospholipids, followed by acetylation of the diradylglycerol products, separation by TLC, and quantitation by gas liquid chromatography (GLC) of the derived FAME gives the diacyl and alkylacyl subclass contents (2–4,7,8). In a variation of this approach (9), the diacyl subclass is first selectively hydrolyzed by a special phospholipase A_1, and the alk-1-enylacyl class is hydrolyzed by exposure to HCl fumes; quantitation of the TLC separated lyso-PL from phospholipase A_1 treatment, the lyso-PL from HCl treatment and the unreacted alkylacyl-PL gives the contents of diacyl, alk-1-enylacyl and alkylacyl subclasses, respectively.

A different approach has been developed (10) in which the total lipid is first reduced with $LiAlH_4$ to yield a mixture of alkylglycerols, alk-1-enyl glycerols and alcohols, which after acetylation with radiolabeled acetic anhydride is resolved into the three lipid molecular classes by TLC and quantitated by radioscanning of the TLC plate. The alkyl and alkenyl ether chain composition is determined by GLC after treatment of the acetyl derivatives with hydrochloric acid in diethyl ether to liberate the aldehyde groups. In a variation (11,12) of the $LiAlH_4$ reduction procedure, the alkenylglycerols are converted to alkyl dioxanes, and the alkylglycerols to isopropylidene derivatives, which then are quantitated by GLC using 1,1-dimethoxyheptadecane and 1-O-heptadecylglycerol, respectively as internal standards carried through the procedure.

Molecular species composition within each of the three subclasses of an individual phospholipid (PL) has been determined by phospholipase C hydrolysis, separation of the diradylglycerols (as such or as acetates) according to their degree of unsaturation by argentation TLC, and identification and quantitation of the molecular species by GLC analysis (with internal standard) of the derived fatty acid methyl esters (FAME), dimethylacetals (DMA) and alkylglycerols (4,7,8,13). More sophisticated procedures for analysis of phospholipid subclasses and their molecular species involve modification of the phospholipid by phospholipase C hydrolysis, followed by chemical derivatization (acetate or benzoate, *tert*-butyldimethylsilyl [*t*-BDMS] or trimethylsilyl [TMS] ether) of the diradylglycerol products and resolution and quantitation of the derivatized molecular species by GLC (14), gas chromatography-mass spectrometry (GC-MS) (15–19) or high pressure liquid chromatography (HPLC) (2,15,16, 20,21,23) with or without prior TLC (22) or HPLC (23) separation of the molecular subclasses.

In a study on changes in platelet lipid composition during storage, the need arose for a relatively simple procedure for analysis of molecular classes that could be used for multisample analysis within a reasonable time period. Therefore, we have developed a chemical method based on two well-known lipid degradative reactions that avoids TLC separation of degradation products. The method will provide the molecular class composition of a phospholipid as well as the overall acyl, aldehyde and alkyl chain composition, but not the radyl chain composition of each class.

In this procedure (Scheme 1), the phospholipid sample is first subjected to acid methanolysis and then to acetolysis (7). Methanolysis produces FAME and DMA from the acid sensitive acyl ester and vinyl ether groups, respectively, and lysoalkyl phosphatidic acids (lysoalkyl-PA)

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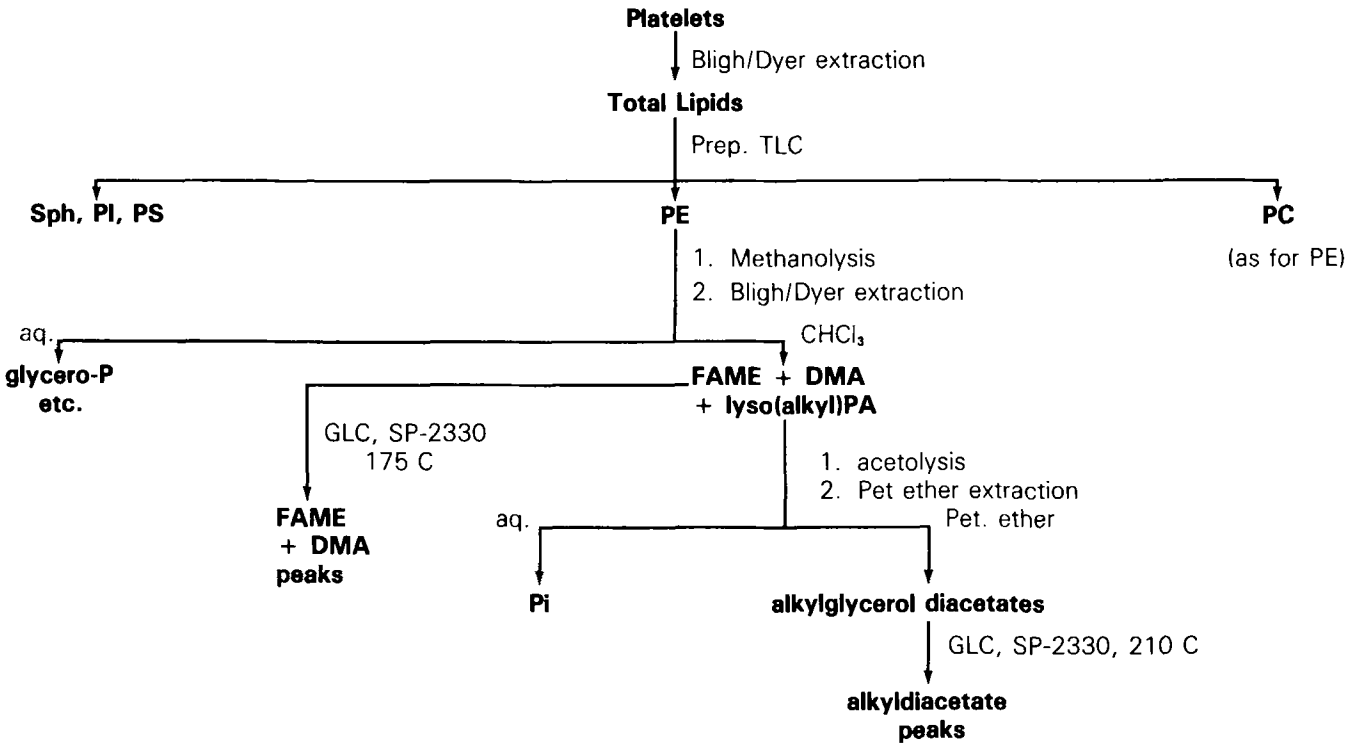
Abbreviations: *t*-BDMS, *tert*-butyldimethylsilyl; CDPA-1, citrate-phosphate-dextrose-adenine; DMA, dimethylacetal; FAME, fatty acid methyl ester; MAGD, monoalkylglycerol diacetate; PA, phosphatidic acid; PAF, platelet activating factor; PRP, platelet-rich plasma; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; TMS, trimethylsilyl; GC, gas chromatography; GLC, gas liquid chromatography; HPLC, high pressure liquid chromatography; MS, mass spectrometry; TLC, thin layer chromatography.



SCHEME 1. Procedure for analysis of molecular subclasses of phospholipids.

from the alkylacyl phospholipids. These products are extracted into chloroform in a Bligh-Dyer partition (24,25), and the FAME and DMA are quantitated by GLC. The lysoalkyl-PA are then derivatized by acetolysis (7) to monoalkyl glycerol diacetates (MAGD) and quantitated by GLC.

In this paper, we demonstrate the feasibility of this methanolysis/acetolysis procedure with authentic standard phospholipids, and its application to the analysis of phospholipid molecular classes and chain type composition in resting platelets from humans.

MATERIALS AND METHODS

All solvents were glass-distilled before use. All other chemicals were of reagent grade unless otherwise specified. Methanolic-HCl (0.6 N) was prepared by diluting 5 ml of concentrated HCl to 100 ml with methanol.

FAME standards were from NuChek (Elysian, MN). DMA were prepared from the sodium bisulfite-adducts of palmitaldehyde and stearaldehyde (K+K Lab Inc., Plainview, NY) by heating at 75 C in a closed screw-capped tube in 4.5 ml of 0.6 N methanolic-HCl for one to two hr followed by addition of 0.6 ml of 8 N aqueous NaOH, further heating at 75 C for one hr, and extraction of the DMA with petroleum ether (25). Purity of the DMA was checked by TLC (Rf, 0.36 in petroleum ether/ethyl ether, 9:1, v/v). GLC analysis showed that the TLC pure palmitaldehyde DMA sample contained ca. 90% 16:0 DMA with 1-2% each of 14:0, 14:1, 16:1, 18:0 and 18:1 DMA; the stearaldehyde DMA sample contained ca. 89% 18:0 DMA with 1-2% each of 14:0, 16:0, 16:1, 18:1 and 20:0 DMA.

MAGD standards were prepared by acetylation (7) of chimyl (16:0), batyl (18:0) and selachyl (18:1) alcohols (Western Chemical Industries Ltd., Vancouver, BC). The MAGD standards were checked for purity and characterized by TLC, GLC and mass spectrometry.

PAF (1-O-hexadecyl-2-acetyl-3-glycerophosphocholine) and plasmalogen phosphatidylethanolamine (PE) (bovine brain) were purchased from Sigma Chemical Co. (St. Louis, MO). Plasmalogen PE was partially hydrogenated with PtO_2 as catalyst (25) to form a mixture of alkylacyl, alkenylacyl and diacyl subclasses. Plasmalogen content was estimated by vinyl ether (iodometric) assay (25).

Preparative and analytical TLC plates were obtained from Whatman Chemical Separation Inc. (Clifton, NJ). The glass columns for GLC were packed with 10% SP 2330 on 100/120 Chromosorb W AW (Supelco, Bellefonte, PA).

Preparation of platelets. Human venous blood was collected from donors (Canadian Red Cross, Ottawa Center, Blood Transfusion Service) into citrate-phosphate-dextrose-adenine (CPDA-1) anticoagulant. The fresh platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 150 × g at 22 C for 15 min. The platelets were pelleted by a second spin at 3000 × g for 10 min, washed three times according to the procedure of Mustard et al. (26) and suspended in Tyrodes buffer (5 ml). The platelet concentration (6-12 × 10^9 cells/ml) was determined by electronic particle counting (Coulter Inc., Hialeah, FL). Differential staining and counting revealed the presence of one lymphocyte per 1600 platelets (0.06%); no neutrophils were detected in the final platelet preparation. One preparation of pooled platelets (preparation 1) and three platelet preparations from separate individuals (preps. 2-4), collected at different times, were analyzed.

Extraction and fractionation of platelet lipids (Scheme 1). Cellular lipids were extracted from fresh platelets by the method of Bligh and Dyer (24) as modified by Kates

(25). Total lipid content of platelets was found to be in range 30–60 μg per 10^8 cells, and total lipid P was 45 ± 9 nmol per 10^8 cells. The choline and ethanolamine glycerophospholipids were separated by preparative TLC using the solvent system chloroform/methanol/ammonia/water (70:30:4:1) (27), with double development. After detection of standard markers with I_2 vapor or Rhodamine 6G, the phosphatidylcholine (PC) and PE bands were eluted from the plates with chloroform/methanol (1:1), partitioned into chloroform, and the chloroform phases were taken to dryness under a stream of nitrogen (25). The residual phospholipids were dissolved in chloroform, to a known volume, and aliquots were taken for P-analysis, TLC to check purity, and subclass analysis. Phospholipid phosphorus was determined by a modification (25) of the method of Bartlett (28).

Determination of diacyl, alkenylacyl and alkylacyl subclass composition of phospholipids (Scheme 1). (1) The methanolysis procedure (25) was as follows: to an aliquot of the chloroform solution containing 1–2 mg of total lipid or 1.3–2.5 μmol of PE or PC fraction in a 20 ml screw-capped (Teflon-lined) test tube was added a known quantity (0.5–1.0 μmol) of internal standard, methyl heneicosanoate (21:0 FAME). The solvent was evaporated under a stream of nitrogen; 4.5 ml of 0.6 N methanolic-HCl was added, and the tube was stoppered and heated for one to two hr at 75 C in a temperature-controlled block heater. After addition of 4.0 ml of water and 4.5 ml of chloroform and centrifugation, the lower chloroform phase was removed, and the upper phase was washed three times with chloroform. The pooled chloroform phases were diluted with benzene and concentrated under a stream of nitrogen. The residual lipids (FAME, DMA, lysoalkyl-phosphatic acid [PA]) were made to a known volume in chloroform, and appropriate aliquots were taken for GLC analysis. The FAME and DMA were analyzed by GLC (in presence of lysoalkyl-PA, which are retained on the column) on a glass column (2 m × 4 mm) of 10% SP2330 at 165 C, 175 C or 195 C depending on the batch of SP2330 used (injector and detector temperatures were 45 C and 50 C, respectively, above the column temperature), and quantitated using the previously added 21:0 FAME as internal standard (25). GLC peak areas were determined by the method of Carroll (29).

(2) The acetolysis procedure (Scheme 1 and ref. 7) was as follows: the remainder of the chloroform phase of the methanolysate described above, containing lysoalkyl-PA + DMA + FAME, after removal of the solvent under a stream of nitrogen was acetolyzed in a screw-capped (Teflon-lined) test tube with 1 ml of acetic acid/acetic anhydride (3:2) at 150 C for 48 hr. To the cooled tube, 4.5 ml of methanol and 0.5 ml of water were added, and the MAGD formed from lysoalkyl-PA, along with remaining FAME and DMA were extracted with three to four portions (5 ml) of petroleum ether. The combined extracts were evaporated under a stream of nitrogen, and the residual lipids were dried in a dessicator over KOH in vacuo and made up to a known volume in chloroform. An aliquot was analyzed for MAGD by GLC at 210 C on a column (2 m × 4 mm) of 10% SP2330 and quantitated using the previously added 21:0 FAME as internal standard (25). Overlapping of FAME with DMA or MAGD may be checked by GLC of the mixture after saponification to remove FAME (25). Replicate analyses included all the steps of the methanolysis and acetolysis procedures.

Molecular subclass calculation. The proportions of molecular classes (mol %) was calculated from the total moles of FAME and DMA obtained by methanolysis and total MAGD obtained by acetolysis, as determined by GLC with 21:0 FAME internal standard, as follows:

$$\text{mol \% diacyl class} = \frac{\Sigma \text{ mol FAME} - \Sigma \text{ mol DMA} - \Sigma \text{ mol MAGD}}{\Sigma \text{ mol FAME} + \text{DMA} + \text{MAGD}} \times 100$$

$$\text{mol \% alkenylacyl class} = \frac{2 \times \Sigma \text{ mol DMA}}{\Sigma \text{ mol FAME} + \text{DMA} + \text{MAGD}} \times 100$$

$$\text{mol \% alkylacyl class} = \frac{2 \times \Sigma \text{ mol MAGD}}{\Sigma \text{ mol FAME} + \text{DMA} + \text{MAGD}} \times 100$$

Recoveries of 1-O-alkyl-2-acyl-, 1-O-alkenyl-2-acyl, or 1,2-diacyl phospholipid classes, as percentages of total PL were checked as follows:

$$\text{mol \% diacyl PL} = \frac{\Sigma \text{ mol FAME} - \Sigma \text{ mol DMA} - \Sigma \text{ mol MAGD}}{2 \times \text{mol PL}} \times 100$$

$$\text{mol \% alkenylacyl PL} = [\Sigma \text{ mol DMA/mol PL}] \times 100$$

$$\text{mol \% alkylacyl PL} = [\Sigma \text{ mol MAGD/mol PL}] \times 100$$

Mol PL was determined by phospholipid phosphorus analysis (25). Statistical evaluation of the data was done by the paired Student's t-test (30).

RESULTS AND DISCUSSION

Standardization of methanolysis/acetolysis procedure. The methanolysis and acetolysis reactions that form the basis of the present method (Scheme 1) were checked for efficiency of separation and recovery of FAME, DMA and MAGD by GLC analysis. After subjecting a known mixture of authentic standard FAME (14:0, 16:0, 16:1, 18:0, 18:1) and DMA (16:0, 18:0) to the methanolysis step, GLC analysis on SP2330 at 175 C with internal standard (21:0 FAME) showed complete resolution of the DMA from the FAME (checked also by GLC analysis of the mixture after saponification to remove FAME) with a recovery of each FAME component in the range 96.9 ± 3.5%; recovery of the DMA components was in the range 99.1 ± 3.1% after correction for the detector response (25) relative to the 21:0 FAME standard (for 16:0 DMA the relative response was 0.722, 0.649 and 0.678 at 165 C, 175 C and 195 C, respectively; and for 18:0 DMA, 0.848, 0.755 and 0.775 for the same temperatures, respectively). Essentially the same recovery of the DMA (99 ± 3%) was obtained at column temperatures of 165 C and 195 C as for column temperature 175 C given above. These results show that under the GLC conditions used, little or no breakdown of the DMA, relative to the internal standard, takes place on a SP2330 column in the temperature range 165–195 C.

GLC analysis on SP2330 at 210 C of a known mixture of authentic standard MAGDs, after subjection to the acetolysis step, showed good separation (ret. rel. to 21:0 FAME: 16:0 MAGD, 4.81; 18:0 MAGD, 7.68; 18:1

MAGD, 8.89) and recovery of each component in the range 99.1 ± 0.5%. The corrected detector response relative to 21:0 FAME thus was close to 100%.

The efficiency of the methanolysis procedure for formation and extraction of the lysoalkyl-PA formed from alkylacyl phospholipids (Scheme 1) was checked with a known amount (27.8 μg P) of standard platelet-activating factor (PAF) (1-*O*-hexadecy-2-O-acetyl-*sn*-glycerophosphorylcholine). Virtually all of the lipid-P (96.6 ± 0.6%) appeared in the chloroform phase as lysoalkyl-PA (Rf, 0.44 and 0.03 on Silica Gel H in solvent systems chloroform/methanol/acetic acid/water, 25:15:4:2, and chloroform/methanol/ammonia/water, 70:30:4:1, respectively) and negligible amounts (2.5 ± 0.1%) were retained in the methanol-water phase of the Bligh-Dyer extraction.

The conditions for the acetolysis procedure (essentially those of Renkonen [7]) were checked by subjecting the chloroform-soluble fraction of the methanolysis products of PAF to the acetolysis procedure (Scheme 1) and analyzing the MAGD formed by GLC on SP2330 at 210 C with the internal standard (21:0 FAME). All of the lysoalkyl-PA phosphorus (99.1 ± 0.6%) was found to be liberated into the aqueous phase of the acetolysate and recovery of the MAGD (1-*O*-hexadecyl glycerol diacetate) was 99.5 ± 0.1%. The optimal time of the acetolysis reaction also was checked with the standard PAF substrate and found to be 48 hr at 150 C, as reported by Renkonen (7).

The methanolysis/acetolysis procedure then was applied to the analysis of samples of unhydrogenated and partially hydrogenated bovine brain plasmalogen PE. Optimal conditions for the acetolysis reaction were found to be the same as for PAF (48 hr at 150 C) and good separation and recovery of the FAME, DMA and MAGD formed was obtained (95 ± 3%). The alkenylacyl subclass content (24.1 ± 0.9 mol %) in the unhydrogenated sample calculated from the GLC data was in good agreement with that calculated from the vinyl ether assay (23.7 ± 1.0 mol %); no alkylacyl subclass was detected by the methanolysis/acetolysis procedure. The contents of diacyl, alkenylacyl and alkylacyl subclasses in the partially hydrogenated sample (75.8 ± 2.8, 7.5 ± 0.3 and 16.6 ± 0.6 mol %, respectively) corresponded well with the respective contents calculated from the vinyl ether assay (76.3 ± 1.8, 7.0 ± 0.8 and 16.7 ± 1.8 mol %).

Analysis of human platelet phospholipid subclasses. The overall phospholipid composition of fresh resting pooled platelets (preparation 1), as determined by P-analysis of TLC-separated components (PC, PE, SPH + PS + PI), was found to be in good agreement with the results reported by other workers (2,5,12,13,31-34) (Table 1).

The methanolysis/acetolysis procedure then was applied to the analysis of molecular subclasses of the PC and PE components. Data are given in Table 1 for one preparation (prep. 1) of platelets from pooled blood and for three preparations from separate individuals (preps. 2-4), collected at different times. The major phospholipid, PC, contained mainly the diacyl class (range for the four preparations, 86-92 mol %) with significant proportions of alkylacyl subclass (range, 7-10 mol %), and only small proportions of alkenylacyl subclass (2-3 mol %). In contrast, PE contained much lower proportions of diacyl subclass (39-60 mol %), somewhat lower proportions of alkylacyl subclass (5-8 mol %) and much higher but variable contents of alkenylacyl subclass (34-55 mol %). The values for diacyl and alkylacyl classes in PC appear to be fairly constant for different platelet preparations and are in reasonable agreement with the corresponding values (82, 94% diacyl; 10, 4.5% alkylacyl) reported by Mueller et al. (2) and Natarajan et al. (12), respectively.

In contrast, the alkenylacyl content of PE was quite variable, ranging from 34% to 55%, the higher value agreeing with that reported by Mueller et al. (2) and the lower value with that of Mahadevapa and Holub (13). The alkenylacyl content of PC, however, was consistently lower than that reported by Mueller et al. (2) but somewhat higher than that of Natarajan et al. (12). Previous workers (5,6,12,13,31,32,35,36) using different analytical procedures also have reported a wide range of values (30-60%) for the alkenylacyl content in human platelet PE and a restricted range (trace to 3%) in platelet PC. The alkenylacyl contents found here (Table 1) thus fall

TABLE 1

Overall Phospholipid Composition and Molecular Subclass Composition of the PC and PE Components of Fresh Resting Platelets[a]

Component and subclass	Present study				Ref. 2	Ref. 12	Ref. 13
	Prep. 1 (n = 3)	Prep. 2 (n = 2)	Prep. 3 (n = 2)	Prep. 4 (n = 2)	(n = 3)	(n = 3)	(n = 4)
SPH + PI + PS	29.6 ± 1.3	N.D.	N.D.	N.D.	32.9 ± 4.1	29.2 ± 2.2	36.8 ± 1.3
PC	38.2 ± 2.7	N.D.	N.D.	N.D.	38.0 ± 1.6	41.0 ± 0.7	39.9 ± 0.8
Diacyl	85.8 ± 2.0	91.9 ± 2.3	91.1 ± 2.3	90.5 ± 2.3	81.8 ± 1.6	94.1	N.D.
Alkenylacyl	1.9 ± 0.4	2.0 ± 0.1	2.7 ± 0.2	2.6 ± 0.2	8.8 ± 2.4	1.4	tr
Alkylacyl	10.4 ± 1.0	6.1 ± 0.4	6.2 ± 0.4	6.9 ± 0.5	9.7 ± 0.3	4.5	N.D.
PE	32.2 ± 1.3	N.D.	N.D.	N.D.	25.3 ± 1.8	26.6 ± 2.5	23.3 ± 1.1
Diacyl	59.7 ± 2.0	38.7 ± 1.0	50.8 ± 1.3	45.9 ± 1.2	36.1 ± 0.3	53.0	N.D.
Alkenylacyl	34.0 ± 1.9	54.8 ± 3.3	44.7 ± 2.7	48.5 ± 2.9	60.4[b]	45.3	32.4 ± 2.8[c]
Alkylacyl	8.0 ± 0.4	6.5 ± 0.4	4.6 ± 0.3	5.6 ± 0.4	3.5 ± 0.1	1.7	N.D.

[a]Values for phospholipid composition (mol % based on phospholipid-P) are given as means ± SD for the number of analyses indicated. Molecular subclass compositions (mol % of PC or PE) are means ± SD for prep. 1 in triplicate but are means ± average deviations for preps. 2-4 done in duplicate.

[b]Calculated by difference (from authors' data [2]).

[c]Calculated from the reported GLC data on DMA (13).

N.D., not determined.

TABLE 2

Summary of Fatty Acid, Aldehyde and Alkyl Ether Chain Composition of Choline and Ethanolamine Phosphoglyceride Components in Fresh Resting Human Platelets[a]

	Choline phosphoglycerides			Ethanolamine phosphoglycerides		
Chain	Acyl	Alkenyl	Alkyl	Acyl	Alkenyl	Alkyl
14:0	tr	N.D.	9.2 ± 3.0	tr		20.8 ± 2.0
14:1	N.D.	N.D.	5.1 ± 1.7	tr		6.2 ± 4.5
16:0	28.4 ± 1.3	50.3 ± 10.0	26.3 ± 1.7	4.0 ± 0.4	18.0 ± 4.0	15.6 ± 3.5
16:1	0.7 ± 0.2	5.9 ± 1.8	N.D.	0.5 ± 0.3	N.D.	N.D.
18:0	13.7 ± 0.4	33.6 ± 11.0	28.2 ± 2.2	16.7 ± 1.6	61.1 ± 2.9	39.2 ± 10.0
18:1	24.0 ± 0.4	10.3 ± 0.4	20.5 ± 3.3	7.2 ± 0.2	14.6 ± 4.0	9.1 ± 6.5
18:2	7.9 ± 0.7	N.D.	N.D.	5.4 ± 0.5	N.D.	6.3 ± 2.0
20:0	1.1 ± 0.04	N.D.	4.0 ± 1.3	0.7 ± 0.2	6.3 ± 2.8	tr
20:1	1.4 ± 0.2	N.D.	tr	0.6 ± 0.2	tr	tr
20:3	1.7 ± 0.2	N.D.		0.8 ± 0.2	N.D.	N.D.
20:4	17.2 ± 1.4	N.D.		48.0 ± 5.5	N.D.	N.D.
22:4	1.2 ± 0.6	N.D.		7.2 ± 1.1	N.D.	N.D.
22:5	1.2 ± 0.6	N.D.		1.2 ± 0.5	N.D.	N.D.
22:6	0.7 ± 0.2	N.D.		6.0 ± 1.0	N.D.	N.D.
Other	0.8[b]	tr[c]	6.7[d]	1.7[b]	tr[c]	2.8[d]

[a]Data are given as mol % (means ± SD, n = 3) for platelet preparations 2, 3 and 4.

[b]Traces of 12:0, 20:5, 22:2 and 24:3 fatty acids also were detected.

[c]Traces of unidentified alk-1-enyl chains also were detected.

[d]Traces of unidentified alkyl chains also were detected.

tr, Less than 0.5%; N.D., not detected.

within the range of values reported (2,5,12,13,31,32,35,36) and may reflect biological variations (due to dietary differences) rather than metholological deficiencies. The alkylacyl content of PE found here (5–8%) was somewhat higher than the values (3.5 and 1.7%) reported by Mueller et al. (2) and Natarajan et al. (12), respectively, but may be due partly to the fact that some of the data of Natarajan et al. (12) were obtained with three-day-old platelets. We have found that stored platelets have lower alkylacyl PE and PC contents than fresh platelets (Vishnubhatla, Kates and Adams, unpublished data).

The fatty acyl, alkyl and alk-1-enyl chain distribution of platelet PC and PE from preparations 2–4 are shown in Table 2. The major fatty acid residues in both PE and PC were 16:0, 18:0, 18:1, 18:2 and 20:4 in general agreement with previous reports (2,5,6,13,31–36). The alkyl ether chains of PC consisted mainly of 14:0, 16:0, 18:0 and 18:1 with small amounts of 14:1, 20:0 and 20:1 (Table 2). Mueller et al. (2) found 16:0, 18:0 and 18:1 as major alkyl ether chains plus small amounts of 20:0 in PC, but Natarajan et al. (12) found only 16:0, 18:0 and 18:1 chains. The major alkyl ether chains found in PE were 14:0, 16:0, 18:0 and 18:1 with lower amounts of 14:1 and 18:2 and traces of 20:0 and 20:1 (Table 2). Natarajan et al. (12), however, found only 16:0, 18:0 and 18:1 alkyl chains in PE.

Analysis of alk-1-enyl chain distribution of the PC indicated that the major chains were 16:0, 18:0 and 18:1 with small amounts of 16:1 (Table 2). Mueller et al. (2) have reported 18:0 (52%) and 16:0 (34%) as major chains with 18:1 and 18:2 as minor components, while Natarajan et al. (12) report the presence only of 16:0, 18:0 and 18:1 chains. PE alk-1-enyl chain composition showed major amounts of 16:0, 18:0 and 18:1 with small amounts of 20:0 (Table 2). Mahadevappa and Holub (13), Mueller et al. (2) and Natarajan et al. (12) found the same major alkenyl chains (16:0, 18:0 and 18:1), but small amounts of 18:2 (2,12), and 20:0 and 20:1 (12) also were reported.

Thus, fatty acyl residues show little variability in composition in the various platelet preparations studied here and elsewhere, but there appears to be more variability in the ether-linked aldehyde and alkyl chain compositions (Table 2) and some inconsistencies with the literature values. However, it should be noted that there are discrepancies also in the alkyl and alk-1-enyl chain compositions reported in the literature (2,12,13). We have observed several unidentified peaks on GLC analysis of the MAGD, which may partly account for some of the discrepancies. Further studies using capillary column GLC-mass spectrometry are necessary to identify these peaks and to check the identity of the MAGD and DMA.

The procedure described here for phospholipid subclass analysis was found to be well-suited for replicate and multisample analysis, on the level of 1–2 μmol, with a reproducibility of ±2–3%, ±5–7%, and ±5–8% for diacyl, alkenylacyl and alkylacyl subclasses, respectively, and within a reasonable time period (7–10 days for 3–4 samples in duplicate). At the same time, the procedure also gives data on the hydrocarbon chain type and chain length for fatty acid, aldehyde and alkyl group constituents of phospholipids. It should prove useful as an adjunct to the more sophisticated procedures for molecular subclass and species analysis of phospholipids by HPLC (2,15,16,20–23) and GC-MS (15–19) methods, to provide a relatively simple assessment of molecular subclasses in a particular phospholipid.

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REFERENCES

1. Snyder, F. (1985) *Med. Res. Rev. 5,* 107-140.
2. Mueller, H.W., Purdon, A.D., Smith, J.B., and Wykle, R.L. (1983) *Lipids 18,* 814-819.
3. Mueller, H.W., O'Flaherty, J.T., and Wykle, R.L. (1982) *Lipids 17,* 72-77.
4. Wood, R., and Snyder, F. (1969) *Arch. Biochem. Biophys. 131,* 478-494.
5. Cohen, P., and Derksen, D. (1969) *Br. J. Haematol. 17,* 359-371.
6. Owen, J.S., Hutton, R.A., Day, R.C., Bruckdorfer, K.R., and McIntyre, M. (1981) *J. Lipid Res. 22,* 423-430.
7. Renkonen, O. (1965) *J. Am. Oil Chem. Soc. 42,* 299-309.
8. Renkonen, O. (1966) *Biochim. Biophys. Acta 125,* 288-309.
9. Tamer, A.E., Recond, M., Fauvel, J., Chap, H., and Douste-Blazy, L. (1984) *Biochim. Biophys. Acta 793,* 213-220.
10. Totani, N. (1982) *Fette Seifen Anstrichm. 84,* 70-73.
11. Su, K.L., and Schmid, H.H.O. (1974) *Lipids 9,* 208-213.
12. Natarajan, V., Zuzarte-Augustin, M., Schmid, H.H.O., and Graff, G. (1982) *Throm. Res. 20,* 119-125.
13. Mahadevappa, V.G., and Holub, B.J. (1972) *Biochim. Biophys. Acta 713,* 73-79.
14. Myher, J.J., and Kuksis, A. (1984) *Can. J. Biochem. Cell Biol. 62,* 352-362.
15. Kuksis, A., Myher, J.J., and Marai, L. (1984) *J. Am. Oil Chem. Soc. 61,* 1582-1589.
16. Kuksis, A., Myher, J.J., and Marai, L. (1985) *J. Am. Oil Chem. Soc. 62,* 762-767.
17. Kuksis, A. (1972) in *Prog. Chem. Fats Other Lipids* (Holman, R.T., ed.) Vol. 12, pp. 1-163, Pergamon Press, New York.
18. Hasegawa, K., and Suzuki, T. (1975) *Lipids 10,* 667-672.
19. Dickens, B.F., Ramesha, C.S., and Thompson, G.A. Jr. (1982) *Anal. Biochem. 127,* 37-48.
20. Patton, G.M., Fasculo, J.M., and Robins, S.J. (1982) *J. Lipid Res. 23,* 190-196.
21. Nakagawa, Y., and Horrocks, L.A. (1983) *J. Lipid Res. 24,* 1268-1275.
22. Blank, M.L., Robinson, M., Fitzgerald, M., and Snyder, F. (1984) *J. Chromatogr. 298,* 473-482.
23. Blank, M.L., Cress, E.A., and Snyder, F. (1987) *J. Chromatog. 392,* 421-425.
24. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biol. Physiol. 37,* 911-917.
25. Kates, M. (1986) in *Techniques in Lipidology: Isolation, Analysis and Identification of Lipids,* revised 2nd edn., pp. 102, 107-108, 124-128, 130-131, 248-250, 274-275, Elsevier Science Publishing, Amsterdam, New York.
26. Mustard, J.F., Perry, D.W., Ardlie, N.G., and Packham, M.A. (1972) *Br. J. Haematol. 22,* 193-204.
27. Skidmore, W.D., and Entenman, C. (1962) *J. Lipid Res. 3,* 471-475.
28. Bartlett, G.R. (1959) *J. Biol. Chem. 234,* 466-468.
29. Carroll, K.K. (1961) *Nature 191,* 377-378.
30. Steel, R.G.D., and Torrie, J.H. (1980) *Principles and Procedures of Statistics,* p. 102, McGraw-Hill, Toronto.
31. Nordöy, A., and Lund, S. (1968) *Scand. J. Clin. Lab. Invest. 22,* 328-338.
32. Marcus, A.J., Ullman, H.L., and Safier, L.D. (1969) *J. Lipid Res. 10,* 108-114.
33. Skeaf, C., and Holub, B.J. (1985) *Biochim. Biphys. Acta 834,* 164-171.
34. Hamid, M.A., Kunicki, T.J., and Aster, R.H. (1980) *Blood 55,* 124-130.
35. Horrocks, L.A. (1972) in *Ether Lipids: Chemistry and Biology* (Snyder, F., ed.) p. 177, Academic Press, New York.
36. Horrocks, L.A., and Sharma, M. (1982) in *Phospholipids* (Hawthorne, J.L., and Ansell, G.B., eds.) p. 51, Elsevier Science Publishing, Amsterdam.

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COMMUNICATIONS

Modification of the Fatty Acid Composition of L1210 Leukemia Subcellular Organelles

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We have examined the extent to which it is possible to modify the fatty acid composition of subcellular organelles of L1210 leukemia cells. A polyunsaturated fatty acid, docosahexaenoic acid, or a monounsaturated fatty acid, oleic acid, were added to the culture media. After 48 hr, the cells were ruptured and the subcellular fractions isolated. Fatty acid analysis revealed that nuclei, mitochondria, plasma membranes and microsomes of the cells grown in media supplemented with docosahexaenoic acid contained increased amounts of polyenoic fatty acids, mean number of double bonds and docosahexaenoic acid compared with cells grown in oleic acid. We conclude that it is possible to experimentally modify the lipids of multiple intracellular structures of L1210 cells by the addition of fatty acids to the growth media.
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It is well-established that it is possible to modify the fatty acid composition of the total cellular phospholipids and plasma membranes of the L1210 murine leukemia cell (1,2) and other neoplastic cells (3,4). Experimental modification can be brought about in vitro by the addition of supplemental fatty acids to the culture media (4,5) or in vivo by lipid-supplementing the diets fed to tumor-bearing host animals (2,3). Normal tissues of animals and humans also can be altered by dietary supplementation (6,7). The biologic implications and therapeutic potential of such alteration recently have been reviewed (8,9). However, much less information is available about the potential for lipid alteration of subcellular organelles such as nuclei, mitochondria and microsomes. Therefore, we have undertaken a study of the extent of fatty acid modification of four intracellular organelles isolated from cells incubated in media supplemented with fatty acids known from previous studies in our laboratory to affect the fatty acid composition of total cellular phospholipids.

METHODS

Fatty acid modification. L1210 murine leukemia cells were grown in suspension culture in medium consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), 5% fetal bovine serum (KC biologicals Inc., Lenexa, KS), with gentamicin sulfate (40 μg/ml) at 37 C in a humidified atmosphere of 5% CO_2/95% air. Modification of the fatty acid composition was accomplished as described (5,10). Briefly, the cells were grown for 48 hr in medium supplemented with 32 μM docosahexaenoic acid (22:6; number of carbon atoms:number of double bonds) or oleic acid (18:1) (NuChek Prep., Inc., Elysian, MN). The fatty acid composition of the nuclear, mitochondrial, plasma membrane and microsome fractions, were determined using gas chromatography as described (2).

Subcellular fractionation. L1210 cells were disrupted and the fractions separated using the Method I of Tsai et al. (11) except for the following modifications: (a) cells suspended in homogenizing fluid were passed through a 25-gauge needle (30 strokes) for disruption. (b) The pellet and supernatants combined from two consecutive centrifugations of homogenate at 1300 × g for one min were utilized. (c) Both discontinuous gradients were made by placing 14 ml of 30% sucrose over 7 ml of 45% sucrose. Aliquots were taken at each step of the procedure and saved for subsequent enzyme activity and DNA content.

Chemical methods. 5′-Nucleotidase was determined through use of radiolabeled substrate (12). Assays of NADPH cytochrome C reductase (13) and succinic acid dehydrogenase activity (14), DNA content (15) and protein (16) also were determined on each relevant fraction.

RESULTS

Characterization of subcellular fractions. Aliquots of each interface and layer of the final gradient were taken for determination of protein, DNA, 5′-nucleotidase, succinic acid dehydrogenase and NADPH cytochrome C reductase. These fractions included the total homogenate, post-nuclei homogenate (supernatants of two 1,300 × g centrifugations), nuclei (pellet of second 1,300 × g centrifugation), membrane (interface between 10% and 30% layers of sucrose gradients after 23,000 × g ultracentrifugations), mitochondria (interface between the 30% and 45% layers plus the 45% layer of sucrose gradients after 23,000 × g ultracentrifugation), microsomes (30% layer) and cytosol (10% layer). The results for the most pertinent seven fractions are shown in Table 1. The nuclear fraction contained 68% of the DNA in the homogenate and 81% of the total DNA recovered in nuclei plus post-nuclear fractions. About half of the protein (54%) was recovered in the cytosol; there were smaller amounts in other fractions, especially the nuclei and mitochondria. 5′-Nucleotidase, a plasma membrane marker for murine cells, was enriched 19-fold in the membrane fraction (10/30% interface) and eight-fold in the microsomes. Succinic acid dehydrogenase, which is a mitochondrial marker, was enriched 17-fold in the mitochondria fraction (30/45% interface plus 45% layer), and three-fold in the microsomes. NADPH cytochrome C reductase, a microsomal marker, was enriched six-fold in the microsomes and 2.5-fold in the plasma membrane fraction. We conclude from this enzymatic and DNA data that our subcellular fractionation procedure yielded fractions that were individually enriched in nuclei, mitochondria, plasma membrane or microsomes.

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TABLE 1

Purification of Subcellular Fractions by Enzyme Marker Analysis

Fraction[a]	Protein	DNA	Marker enzyme specific activity		
			5'-Nucleotidase	Succinic acid dehydrogenase	NADPH Cyto. C Red.
	mg/10^8 cells	mg/10^8 cells	nmol/min per mg protein		
Homogenate	13.4 ± 0.9	2.5 ± 0.5	15.2 ± 2.7	4.35 ± 1.06	—
Post-nuclei homogenate	11.0 ± 0.7	0.4 ± <0.1	15.6 ± 2.5	4.20 ± 1.63	5.9 ± 1.2
Nuclei	1.2 ± 0.1	1.7 ± 0.1	2.9 ± 0.7	0.29 ± 0.15	5.2 ± 3.9
Plasma membrane	0.08 ± 0.01	0.08 ± 0.04	300.0 ± 57.7	3.50 ± 1.89	15.6 ± 3.1
Mitochondria	0.87 ± 0.07	0.04 ± 0.03	10.2 ± 2.2	69.50 ± 15.50	2.6 ± 1.9
Microsome	0.13 ± 0.03	0.20	129.6 ± 31.0	14.3 ± 9.13	38.3 ± 23.3
Cytosol	5.9 ± 0.4	0.03	0	0.4 ± 0.2	6.8

[a]Values are mean and SE of selected fractions after 1,300 × g centrifugation to remove nuclei.

TABLE 2

Fatty Acid Composition of L1210 Subcellular Fractions

Fatty acid	Fatty acid composition (%)[a]							
	22:6-Supplemented				18:1-Supplemented			
	Nuclei	Mitochondria	Plasma membrane	Microsomes	Nuclei	Mitochondria	Plasma membrane	Microsomes
Individual acids[b]								
16:0	18.1 ± 1.2	16.1 ± 0.7	18.2 ± 0.3	20.9 ± 1.5	10.8 ± 0.2	12.8 ± 0.4	11.5 ± 1.4	13.6 ± 3.0
16:1	4.3 ± 0.4	N.D.	N.D.	N.D.	5.5 ± 1.3	0.6 ± 0.6	2.2 ± 0.8	1.2 ± 1.2
18:0	13.9 ± 0.8	2.1 ± 2.1	20.1 ± 0.1	28.0 ± 1.9	5.2 ± 0.1	4.8 ± 0.5	13.4 ± 2.4	8.6 ± 0.6
18:1	26.2 ± 1.9	33.7 ± 2.3	23.1 ± 0.8	28.8 ± 1.4	63.2 ± 4.3	65.6 ± 2.1	58.1 ± 2.2	63.5 ± 2.4
18:2	2.6 ± 0.4	N.D.	0.4 ± 0.2	1.0 ± 0.5	2.6 ± 0.3	4.4 ± 2.6	1.1 ± 0.4	1.3 ± 1.3
18:3	1.2 ± 0.3	<0.1 ± <0.1	N.D.	0.4 ± 0.2	3.6 ± 0.6	3.2 ± 0.5	4.4 ± 0.3	3.8 ± <0.1
20:4	3.3 ± 0.2	3.6 ± 0.2	4.0 ± 0.3	1.6 ± 0.9	3.4 ± 0.2	3.2 ± 0.6	5.4 ± 0.5	4.3 ± 0.9
20:5	0.2 ± <0.1	1.8 ± 0.2	1.4 ± 0.2	0.9 ± 0.5	0.1 ± 0.1	0.3 ± <0.1	0.4 ± 0.2	0.2 ± <0.1
22:5	0.3 ± 0.2	0.9 ± 0.2	1.5 ± 1.0	0.2 ± 0.2	0.6 ± <0.1	1.3 ± 0.1	1.8 ± 0.5	0.5 ± <0.2
22:6	26.2 ± 2.4	40.8 ± 1.4	30.3 ± 1.2	17.4 ± 1.6	1.2 ± 0.3	1.8 ± 0.8	1.2 ± 0.3	0.4 ± 0.2
Other[c]	3.8	0.1	1.1		3.7	2.2	0.7	
Classes[d]								
Saturates	33.6 ± 1.7	19.1 ± 2.3	39.0 ± 0.5	49.1 ± 0.4	18.6 ± 1.5	21.4 ± 2.4	25.2 ± 3.7	23.7 ± 4.4
Monoenoics	30.5 ± 2.3	33.7 ± 2.3	23.1 ± 0.8	28.8 ± 1.4	68.7 ± 3.0	66.2 ± 2.4	60.2 ± 2.2	64.7 ± 2.5
Polyenoics	34.8 ± 3.4	47.2 ± 1.1	37.6 ± 0.2	21.8 ± 1.7	12.1 ± 1.4	14.5 ± 2.5	14.6 ± 2.0	11.1 ± 3.0
Mean no. double bonds	2.1 ± 0.1	3.1 ± 0.1	2.4 ± <0.1	1.5 ± 0.1	1.1 ± <0.1	1.1 ± <0.1	1.2 ± 0.1	1.0 ± 0.1
Mean chain length	18.4 ± <0.1	19.4 ± <0.1	18.9 ± <0.1	18.3 ± 0.1	17.6 ± 0.1	17.8 ± <0.1	18.0 ± <0.1	17.7 ± 0.2

[a]Mean ± SE of determinations made on individual samples from three to four separate experiments.

[b]Among the individual fatty acids, the amounts of 16:0, 18:0, 18:1, 18:3 and 22:6 for the nuclei, 16:0, 18:1, 18:3, 20:5, and 22:6 for the mitochondria, 16:0, 18:0, 18:1, 18:3, 20:5 and 22:6 for the membranes and 18:0, 18:1, 18:3 and 22:6 for the microsomes are significantly different for 22:6- vs 18:1-supplemented cells ($p < 0.05$).

[c]Includes small amounts of 12:0, 14:0, 20:3, 22:4 and unidentified fatty acids.

[d]Significantly different values ($p < 0.05$) for 22:6- vs 18:1-supplemented cells were found for the amounts of saturates, monoenoics, polyenoics, mean number of double bonds and mean chain length for the nuclei, monenoics, polyenoics, mean number of double bonds and mean chain length for the mitochondria, saturates, monoenoics, polyenoics, mean number of double bonds and mean chain length for the plasma membranes and saturates, monoenoics, polyenoics, mean number of double bonds and mean chain length for the microsomes.

N.D., not detected.

Fatty acid composition. We have shown that the medium fatty acid supplementation techniques used in this paper result in enrichment of the L1210 cellular phospholipids in the supplemented fatty acid (5,17). In this study, we determined the fatty acid composition of selected subcellular fractions of the 22:6- and 18:1-enriched leukemia cells to allow comparison of fatty acid enrichment (Table 2). There was considerable modification of the fatty acid composition of all four fractions studied. Each of the subcellular fractions of the cells grown in 22:6-supplemented media were enriched in 22:6. This enrichment ranged from 17% of the total fatty acids in the microsomes and was the greatest, 40%, in the mitochondria. This compares with only 0–2% 22:6 in the 18:1-supplemented cells. Conversely, the cells grown in 18:1-supplemented media contained 58–66% 18:1 compared with only about half that much in the organelles of the 22:6-supplemented cells. The organelles of the

22:6-supplemented cells contained more palmitic acid (16:0) but less linolenic acid (18:3). These changes in the individual acids resulted in more polyunsaturation and greater mean number of double bonds in the 22:6-supplemented cells and more monounsaturation in the 18:1-supplemented cells. Noteworthy was the observation that the mitochondria contained only 2% and 5% stearic acid (18:0) compared with 20% and 13% in plasma membranes, regardless of fatty acid supplementation.

DISCUSSION

The important finding of this study is that it is possible to modify the fatty acid composition of multiple intracellular organelles. It is well known that substantial differences in the composition of total cellular phospholipids and of plasma membranes can be produced in vivo and vitro (8). In contrast, there is only limited information on experimental alteration of other subcellular fractions. For example, there has been a study on the modification of the lipids of nuclei. Awad and Spector reported that the nuclear lipids of the Ehrlich ascites tumor cell were altered by feeding the tumor-bearing mice diets rich in either coconut or sunflower oil (18). These changes were qualitatively similar to those that the investigators had reported earlier in the plasma membranes of the same cell type, but were quantitatively somewhat less (19). There also have been reports regarding the experimental modification of the cellular microsomes of human Y79 retinoblastoma cells (4,20). There was considerable enrichment of the fatty acids of microsome lipids with 18:1, arachidonic acid (20:4), 18:3 or 22:6 added to the culture medium. However, these studies did not separate microsomes from plasma membranes so that the crude microsomal fraction that was studied contained both endoplasmic reticulum and plasma membranes. In our study, the extent of enrichment of the L1210 microsomes grown in 22:6 was only 17% of the total fatty acid compared with a greater percent enrichment of 26–41% in the other subcellular fractions. It is interesting to note that this is similar to the 15% enrichment of microsomal membranes of the Y79 cells (20). These studies taken together suggest that the endoplasmic reticulum contained in the microsomal fraction may be perturbed by these fatty acyl substitutions to a lesser extent than plasma membranes.

Dietary-induced alteration in mitochondrial fatty acids of the kidney, liver, heart and other tissues of normal animals also has been demonstrated (21,22). Similarly, the addition of linoleic acid (18:2) supplements to the medium of cultured mouse fibroblasts resulted in an increase in the mitochondrial linoleic acid (18:2) from not detectable to 33% of the total fatty acids (23). In our study, the degree of insertion of the supplemented fatty acid was somewhat greater in the case of the mitochondria as compared to the nuclei. This could be related to the active role of the mitochondria in oxidative metabolism. We have demonstrated that multiple organelles of the L1210 cell can be modified experimentally with respect to fatty acid composition. The alterations of separated fractions are generally similar. However, the extent of polyunsaturated fatty acid insertion into microsomes was somewhat less, and into mitochondria somewhat more, than plasma membranes and nuclei. The changes that we were able to bring about in the L1210 subcellular fractions in tissue-cultured cells demonstrate that our model can be utilized to study the effects of lipid modification on multiple cell structures.

It is known that 18:1-enriched cells are similar in fatty acid composition and physical properties to unmodified L1210 cells (5). These 18:1 cells, therefore, represent a control cell for comparison with the polyunsaturated-rich cells. We did not study the organelles of unmodified L1210 cells, because such a comparison of modified and unmodified cell fractions would not take into account that only one of the cell types would have been exposed to exogenous fatty acid. We studied intact organelles. It is likely that the major specific site of the fatty acid alteration is the phospholipid-rich membranes of the organelles. However, it would be technically difficult to obtain a pure membrane fraction from the organelles in sufficient quantities for analysis.

These results demonstrate that experimental fatty acid alteration affects more than just plasma membranes exposed directly to the lipids in the supplemented medium. Intracellular structures including mitochondria and nuclei also are modified. It is possible that the fatty acid changes that we have found might be partially or fully compensated by changes in content or type of other lipids of the organelle. However, such was not the case for plasma membranes (8,9). The general nature of the changes provides a rationale for resultant broad functional changes within the cell. Even though most of the biologic effects of fatty acid modification described to date can be related to plasma membranes (8,9), it seems likely that there are also undiscovered consequences relating to mitochondria or nuclear function.

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REFERENCES

1. Burns, C.P., Luttenegger, D.G., Wei, S.L., and Spector, A.A. (1977) *Lipids 12*, 747–752.
2. Burns, C.P., Luttenegger, D.G., Dudley, D.T., Buettner, G.R., and Spector, A.A. (1979) *Cancer Res. 39*, 1726–1732.
3. Liepkalns, V.A., and Spector, A.A. (1975) *Biochem. Biophys. Res. Commun. 63*, 1043–1047.
4. Yorek, M.A., Strom, D.K., and Spector, A.A. (1984) *J. Neurochem. 42*, 254–261.
5. Guffy, M.M., Rosenberger, J.A., Simon, I., and Burns, C.P. (1982) *Cancer Res. 42*, 3625–3630.
6. Burns, C.P., Rosenberger, J.A., and Luttenegger, D.G. (1983) *Ann. Nutr. Metab. 27*, 268–277.
7. Lee, T.H., Hoover, R.L., Williams, J.D., Sperling, R.I., Ravalese, J. III, Spur, B.W., Robinson, D.R., Corey, E.J., Lewis, R.A., and Austin, K.F. (1985) *N. Engl. J. Med. 312*, 1217–1224.
8. Burns, C.P., and Spector, A.A. (1987) *Lipids 22*, 178–184.
9. Spector, A.A., and Burns, C.P. (1987) *Cancer Res. 47*, 4529–4537.
10. Guffy, M.M., North, J.A., and Burns, C.P. (1984) *Cancer Res. 44*, 1863–1866.
11. Tsai, C., Chen, K., and Canellakis, E.S. (1985) *Biochim. Biophys. Acta 401*, 196–212.
12. Avruch, J., and Wallach, D.F.H. (1971) *Biochim. Biophys. Acta 233*, 334–347.
13. Sottocasa, G.L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967) *J. Cell Biol. 32*, 415–438.

14. Johnston, K.H., and Gotschlich, E.C. (1974) *J. Bacteriol. 119*, 250–257.
15. Cattolico, R.A., and Gibbs, S.P. (1975) *Anal. Biochem. 69*, 572–582.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265–275.
17. Burns, C.P., and North, J.A. (1986) *Biochim. Biophys. Acta 888*, 10–17.
18. Awad, A.B., and Spector, A.A. (1976) *Biochim. Biophys. Acta 450*, 239–251.
19. Awad, A.B., and Spector, A.A. (1976) *Biochim. Biophys. Acta 426*, 723–731.
20. Hyman, B.T., and Spector, A.A. (1982) *J. Neurochem. 38*, 650–656.
21. Stubbs, C.D., and Smith, A.D. (1984) *Biochim. Biophys. Acta 779*, 89–137.
22. McMurchie, E.J., Gibson, R.A., Charnock, J.S., and McIntosh, G.H. (1986) *Lipids 21*, 315–323.
23. Gilmore, R., Cohn, N., and Glaser, M. (1979) *Biochemistry 18*, 1042–1049.

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Composition and Physical State of Phospholipids in Calanoid Copepods from India and Norway

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The fatty acid composition and physical state of isolated phospholipids obtained from marine copepods collected on the Southwest coast of India *(Calanus ssp.)* and the West coast of Norway *(Calanus finmarchicus)* were investigated to compare the adaptation of membrane lipids with seawater temperatures. Phospholipid vesicles obtained from the tropic copepods proved more rigid than those from *C. finmarchicus*, as assessed by diphenylhexatriene fluorescence polarization techniques. In each case, there were two breaks present on the fluorescence polarization vs 1/T plots, suggesting that the onset and completion of phase separation to occurred above 0 C. For the tropic copepods, the onset of phase separation roughly corresponded to the ambient water temperature, while for *C. finmarchicus* some discrepancies were observed, depending on the time of the year. Phospholipids in copepods from both habitats contained more than 50% unsaturated fatty acids, the animals from Norway containing slightly higher amounts. The data indicate an adaptation of membranes to temperature.
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Most of our knowledge concerning adaptation of the composition and physical state of membranes to temperature in higher eukariotic systems is derived from observations made on freshwater organisms (1,2). The majority of these investigations demonstrate an inverse relationship between the unsaturation of the constituent phospholipids and temperature (1,3–6), and also a varying degree of homeoviscosus response of membrane physical state to the temperature (7,8). Fatty acids are regarded as the most important factors controlling the physical state of these structures. A major difference between freshwater and marine species is the high level of long-chain polyunsaturated fatty acids in the latter. This should render their membranes more fluid. Based on the data available (2,9,10), the question remains whether they are able to control fatty acid composition according to temperature, by homeoviscosus adaptation. The oil sardine, *Sardinella longiceps,* responded by an increase in the level of docosahexaenoic acid and a decrease of saturated fatty acids when the temperature decreased from 30–31 C to 25–26 C from summer to winter (11). Absence of seasonal variation of phospholipid fatty acids was noted with two marine clam species (12) and with shrimp (13) as well as with *Porphyra yezoensis* exposed to cold for a prolonged time (14). However, in these investigations there was no direct determination of the effect of temperature on the fluidity of membrane phospholipids. In this study, the fatty acid composition and the physical state of phospholipids obtained from marine calanoid copepods collected, respectively, from tropic and temperate seas was investigated.

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Abbreviations: BHT, butylated hydroxytoluene; DPH, 1,6-diphenyl 1,3,5-hexatriene; P, fluorescence polarization.

MATERIALS AND METHODS

Animals. Calanus finmarchicus were collected at the West coast of Norway, at Austevoll (60°7′N, 5°13′E), and Stangvik Fjord (62°48′N, 8°26′E), on April 13, and November 26, 1984, respectively. The water temperature was near 10 C in both cases. Calanoid copepods *Calanus ssp.)* also were collected on the West coast of India. One sample originated from the coastal waters off Bombay, collected one km offshore of the Gateway of India (19°00′N, 72°45′E), on February 16, 1981. Another sample was collected at Dona Paula, Goa (15°N, 72°48′E), on January 20, 1986. The surface temperatures were 26 C and 25 C, respectively. No species were identified from the samples collected in India. The copepods originating from the Bombay area were large specimens resembling the size of *C. finmarchicus* Stage V, while those collected at Dona Paula were considerably smaller. The hauls of *C. finmarchicus* were almost 100% copepods, while those made in India were 90–95% copepods with some contaminating decapod larvae.

Analysis of lipids. The animals were fixed in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) and frozen until transferred to the laboratory. The Folch procedure (15) was used to extract the total lipids. Phospholipids were separated by silicic acid column chromatography using chloroform to remove the neutral lipids and methanol to obtain the phospholipid fraction. The polar head group composition of the latter was determined according to Rouser et al. (16).

Phosphatidylcholine and phosphatidylethanolamine were separated for further analysis by preparative thin layer chromatography using chloroform/methanol/water (65:25:4, v/v/v) as solvent. Identification of the spots was by comparing the R_f values to those of known standards (Supelco, Bellefonte PA). The spots were detected by spraying the plates with 0.05% 8-anilino-1-naphtalene sulfonic acid in 50% methanol and viewed under UV light. Total or individual phospholipids were transmethylated in the presence of 5% HCl in absolute methanol at 80 C in sealed vials for 2.5 hr. A Hitachi 263-80 type gas chromatograph connected to a Hitachi M263 data processor was used to separate the fatty acid methyl esters. The polar phase was 10% Carbowax 20M on 100–120 mesh Supelcoport in 2 m long stainless steel columns (3 mm i.d.). The oven temperature was programed to rise linearly from 180 to 215 C, at 1 C/min. Each run was made in triplicate, and the error was not more than 1% in the case of the major fatty acids such as 22:6(n-3).

Fluorescence polarization. Phospholipid vesicles were prepared and labeled with 1,6-diphenyl 1,3,5-hexatriene (Sigma Chemical Co., St. Louis, MO) as described by Montaudon et al. (17). A Perkin Elmer Model 44A fluorescence spectrophotometer equipped with a polarization accessory and fitted with a temperature regulation unit was used for the measurements. The excitation wavelength was 370 nm, and the fluorescence emission

was monitored at 420 nm. Fluorescence polarization (P) was calculated from the equation, $P = I_{vv} - I_{vh}/I_{vv} + I_{vh} \cdot Z$, in which I_{vv} and I_{vh} are the fluorescence intensities measured with emission analyzer parallel or perpendicular, respectively, to the polarization of the detection system for vertically and horizontally polarized light.

RESULTS AND DISCUSSION

Figure 1 presents a Vant Hoff's representation of 1,6-diphenyl 1,3,5-hexatriene (DPH) fluorescence polarization, P, of vesicles of phospholipids prepared from marine calanoid copepods collected either in the North Atlantic or in tropic seas. Lower P-values represent more fluid structures. From these data, it may be inferred that the most fluid membranes were present in *C. finmarchicus* collected in the spring and the most rigid ones in the copepods inhabiting the tropic seas. *C. finmarchicus* sampled in early fall revealed values in between these two extremes. In addition, there are two distinct breaks in the curves on the P vs 1/T plots. The break at the higher temperature indicates onset, while that at the lower temperature indicates completion of phase separation of these phospholipids. The sea water temperature at the time of collecting *C. finmarchicus* was about 10 C but varied between 2 and 20 C during the year. The temperature in the tropic seas was about 25–26 C at the time of sample collecting and varied from 25 to 30–31 C during the year. The temperature at the onset of phase separation of phospholipids from each sample was close to the temperature at which the organism lived, except in the *C. finmarchicus* collected in the fall. However, it should be remembered that the above values for phospholipid vesicles might be modulated if other membrane constituents (proteins, sterols, etc.) also were present.

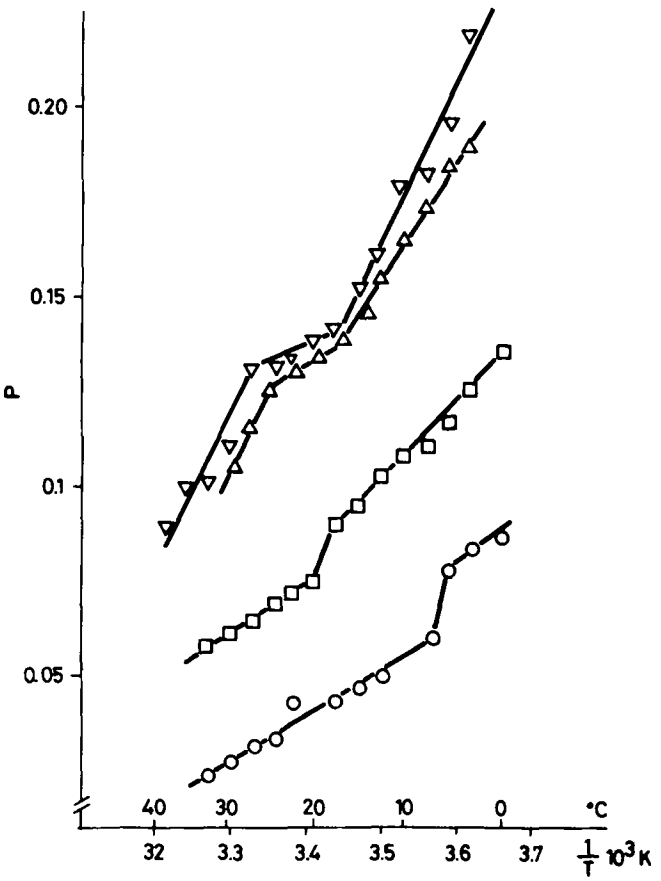

FIG. 1. Temperature dependency (1/T) of DPH fluorescence polarization (P) in phospholipids of marine calanoid copepods. *Calanus spp.* were collected at Dona Paula, Goa (—∇—) and Bombay (—△—) at the Southwest coast of India, while *C. finmarchicus* were collected at the West coast of Norway in the fall (—□—) and in the spring (—○—), respectively.

The observation that the temperature at onset of the phase separation of the membrane lipids coincides with growth temperature has been made with other poikilotherms (10,18–20), but it is not documented as a general phenomenon (7,21). Cossins and Prosser (18) reported that the onset of phase separation of phospholipids from synaptosomal membranes of arctic sculpin adapted to 0 C occurred at 5 C while that for goldfish acclimated at 5 C occurred at 10 C. It can be inferred from Figure 1 of Prosser and Cossin's paper that phospholipids of synaptosomal membranes of goldfish adapted at 25 C show phase separation around the growth temperature (18). In a current study of liver phospholipids of the carp, *Cyprinus carpio L.*, we found that the onset of phase separation of phospholipids of summer-adapted fish occurred around 25 C, while that of winter-adapted fish occurred around 6 C (unpublished observations). In earlier work on the freshwater copepod *Cyclops vicinus*, we also found that phase separation temperatures were similarly related to the actual growth temperature (10). Although the water temperature at the time of collecting *C. finmarchicus* was the same in the fall and the spring (10 C), the higher polarization value and phase separation temperature of phospholipids of the fall sample may suggest that the fall specimens retained a "summer" state in their lipids.

Whether marine species similar to *C. vicinus* can regulate the physical state of their phospholipids according to the temperature or whether they lack this property, like the freshwater crustacean *Daphnia magna*, requires further investigation. Some freshwater crustaceans and fish are exposed to fluctuation in their environmental temperature. In cases of tropic seas, this is less pronounced. Judging from the temperature range at which the phase separation occurs, it may be inferred that *C. finmarchicus* can tolerate less changes in the water temperature than the copepods in the tropic seas.

Spring-collected *C. finmarchicus* did not survive exposure to 20 C longer than two hr, and copepods collected at Dona Paula lost their swimming activity but did not die when exposed to 17 C for six hr (unpublished observations). Because the former can be regarded as a cold stenothermic and the latter as a warm stenothermic species, it is tempting to speculate that this is at least partially related to the phase behavior of their membranes.

Table 1 shows that the above differences in the phase behavior are not easily explained by the fatty acid composition of the phospholipids. An inverse relationship between environmental temperature and fatty acid unsaturation also was observed in this case. This was due mainly to a higher level of docosahexaenoic acid in phospholipids of *C. finmarchicus*. Despite these differences,

TABLE 1

Fatty Acid Composition (mol %) in Phospholipids of Calanoid Copepods

Species	*Calanus spp.*		*C. finmarchius*	
Origin	India[a]	India[b]	Norway[c]	Norway[d]
Water temperature (°C):	25	26	10	10
14:0	4.7	3.9	3.4	2.4
14:1	0.2	0.1	0.5	0.1
15:0	0.2	0.3	0.7	0.2
15:1	0.2	tr	0.1	tr
16:0	19.6	16.0	16.5	15.2
16.1	1.4	1.6	0.3	0.7
16:2	0.9	0.3	0.1	0.3
16:3	1.3	0.2	0.7	0.1
18:0	8.7	4.0	2.0	0.8
18:1	4.5	3.7	2.9	4.5
18:2	1.5	1.5	0.8	0.6
18:3	1.7	1.8	0.7	tr
18:4	0.3	2.5	1.9	0.2
20:1	tr	0.2	0.1	1.2
20:4	2.5	4.5	1.2	1.9
20:5	15.7	25.2	24.1	30.2
22:3	0.5	0.2	0.2	0.2
22:4	0.2	0.1	0.2	0.3
22:5	2.4	2.3	0.4	0.5
22:6	33.4	31.2	42.9	40.0
Sat/unsat	0.49	0.31	0.29	0.22
Total polyen(%)	55.0	66.1	71.0	73.8

[a]Bombay.
[b]Dona Paula, Goa.
[c]Spring.
[d]Fall.

TABLE 2

Composition of Phospholipids (% wt) in Calanoid Copepods

Species:	*Calanus spp.*	*Calanus finmarchius*
Origin:	India[a]	Norway[b]
Phosphatidic acid	3.6	10.1
Phosphatidylserine	3.1	8.9
Phoaphatidylinositol	3.9	6.4
Lysophosphatidyl-ethanolamine	—	5.2
Lysophosphatidyl-choline	6.8	1.1
Sphingomyelin	3.9	7.6
Phosphatidylcholine	35.6	29.3
Phosphatidyl-ethanolamine	28.9	22.3
Cardiolipin	6.8	9.8

[a]Dona Paula, Goa.
[b]Spring.

TABLE 3

Fatty Acid Composition (mol %) of Phosphatidylcholines and Phosphatidylethanolamines in Calenoid Copepods

Phospholipid	Phosphatidyl-choline		Phosphatidyl-ethanolamine	
Origin	India[a]	Norway[b]	India[a]	Norway[b]
14:0	3.6	2.5	1.0	0.5
14:1	tr	tr	tr	tr
15:0	0.4	0.1	tr	tr
16:0	20.9	10.2	20.4	24.6
16:1	1.0	1.1	0.4	0.1
18:0	2.6	0.2	12.4	2.2
18:1	6.4	2.6	2.6	4.3
18:2	1.8	0.7	1.3	1.3
18:3	2.0	0.2	0.9	0.3
18:4	2.4	1.7	0.5	1.5
20:3	tr	tr	tr	tr
20:4	4.9	5.0	6.0	3.3
20:5	21.9	42.8	14.3	9.8
22:4	0.7	tr	0.3	0.7
22:5	2.2	0.5	3.0	1.6
22:6	28.2	32.0	36.7	50.00
Sat/unsat	0.38	0.15	0.51	0.38
Total polyen(%)	62.0	82.0	61.0	67.0

[a]*Calanus ssp.*, Dona Paula, Goa.
[b]*C. finmarchicus*, spring.

the spring-collected *C. finmarchicus* and the copepods collected at Dona Paula showed similar saturated to unsaturated ratios but great differences in the phase behavior of their phospholipids. Moreover, the two samples of copepods from the tropic seas showed differences in phospholipid fatty acid compositions as well as in the saturated to unsaturated fatty acid ratios, although the P-values and the phase separation temperatures were almost identical (Fig. 1). Thus, it is highly probable that control occurs at a level beyond the overall distribution of fatty acids in phospholipids. Table 2 shows that the phospholipids of copepods from tropic seas were poorer in sphingomyelin and phosphatidic acid, and richer in phosphatidylethanolamine than those in *C. finmarchicus*. Phosphatidylcholines in spring-collected *C. finmarchicus* contained more polyunsaturated acids (82% vs 62%) and had a lower saturated-to-unsaturated fatty acid ratio (0.15 vs 0.38) than those of the tropic copepods (Table 3). Even though phospholipids were not separated according to molecular-species composition, one could expect that diunsaturated phospholipids would be present whenever the level of total unsaturated fatty acids exceeds 50 mol %. As shown in Table 3, the phosphatidylcholines and phosphatidylethanolamines were richer in diunsaturated phospholipids than were the phospholipids of copepods in the tropic seas (32% vs 12% and 17% vs 11%, respectively), and this could explain the observed differences in the P-values (Fig. 1). Because the phase separation temperature of 1-palmitoyl,2-docosahexaenoyl phosphatidylcholine is about −10 C (22) and that of diunsaturated molecules is even lower, phospholipids of marine copepods should exhibit lower phase-separation temperatures than those observed. Phospholipids of these copepods behave similarly to those of the bovine retinal rod outer segment membranes. Although the latter are as rich in polyenes as the phospholipids of copepods investigated here, they too contain fair amounts of supraenes (dipolyunsaturated phospholipids) (23,24) and exhibit phase-separation temperature between 15 and 5 C (25).

It has been proposed that this results from a precise balance between disaturated and diunsaturated phospholipid molecules (25). The differences demonstrated in physical parameters of phospholipid vesicles indicate an adaptation of membrane physical states to temperatures.

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REFERENCES

1. Hazel, J., and Prosser, C.L. (1974) *Physiol. Rev. 54,* 620-667.
2. Dutta, H., Das, A., Das, A.B., and Farkas, T. (1985) *Comp. Biochem. Physiol. 81B,* 341-347.
3. Hazel, J. (1979) *Am. J. Physiol. 236,* 91-101.
4. Farkas, T., and Csengeri, I. (1976) *Lipids 11,* 401-407.
5. Wodtke, E. (1978) *Biochim. Biophys. Acta 529,* 280-291.
6. Van den Thillart, G., and Bruin, G. (1981) *Biochim. Biophys. Acta 640,* 439-447.
7. Cossins, A.R., Christiansen, J., and Prosser, C.L. (1978) *Biochim. Biophys. Acta 511,* 442-454.
8. Cossins, A.R., and Prosser, C.L. (1982) *Biochim. Biophys. Acta 687,* 303-309.
9. Farkas, T. (1979) *Comp. Biochem. Physiol. 64B,* 71-76.
10. Farkas, T., Nemecz, Gy., and Csengeri, I. (1984) *Lipids 19,* 436-442.
11. Gopakumar, K. (1973) *J. Mar. Biol. Assoc. (India) 14,* 2-5.
12. Beninger, P.G., and Stephan, G. (1985) *Comp. Biochem. Physiol. 81B,* 591-601.
13. Bottino, N.R., Gennity, J., Lilly, M.L., Simons, E., and Finne, G. (1980) *Aquaculture 19,* 139-148.
14. Kayama, M., Iijima, N., Kuwahara, M., Sado, T., Araki, S., and Sakurai, T. (1985) *Bull. Jpn. Soc. Sci. Fish. 51,* 687.
15. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-507.
16. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) *Lipids 5,* 494-496.
17. Montaudon, D., Robert, J., and Canguilhem, B. (1984) *Biochem. Biophys. Res. Commun. 119,* 396-400.
18. Cossins, A.R., and Prosser, C.L. (1978) *Proc. Natl. Acad. Sci. USA 75,* 2040-2043.
19. Janoff, A.S., Gupte, S., and McGroaty, E.J. (1980) *Biochim. Biophys. Acta 598,* 641-644.
20. Durairay, G.I., and Vijakumar, I. (1984) *Biochim. Biophys. Acta 770,* 7-14.
21. Cossins, A.R., and Prosser, C.L. (1982) *Biochim. Biophys. Acta 687,* 303-309.
22. Coolbear, K.P., Redde, C.B., and Keough, K.M.W. (1983) *Biochemistry 22,* 1466-1473.
23. Milajanich, G.P., Sklar, L.A., White, D.L., and Dratz, E.A. (1979) *Biochim. Biophys. Acta 552,* 294-306.
24. Aveldano, M.I., and Bazan, N.G. (1983) *J. Lipid Res. 24,* 620-627.
25. Sklar, L.A., Miljanich, G.P., Brusten, S.L., and Dratz, E.A. (1979) *Biochemistry 254,* 9583-9591.

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Enhanced Survival to Endotoxin in Guinea Pigs Fed IV Fish Oil Emulsion

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Improved survival to endotoxin has been demonstrated in rats pretreated with cyclooxygenase inhibitors or made essential fatty acid deficient, implying that excessive ω6 fatty acids, possibly through their eicosanoid products, contribute to mortality. Following endotoxin administration, we also have shown improvement in survival with oral diets supplemented with fish oil. This study sought to explore whether parenteral fish oil ameliorates the adverse impact of endotoxin.

Male Hartley-strain guinea pigs were obtained at a body weight of 500 g and fed a normal laboratory diet. Central venous lines through which the animals received either a 10% safflower oil emulsion (n = 11) or a 10% fish oil emulsion (n = 11) during two, 24-hr periods separated by two days were inserted. Two days after the second infusion, endotoxin (0.35 mg/100 g b.w.), was given intraperitoneally, and survival was noted. The animals received a total of 25.4 g of IV fat per kg b.w., including 5.3 g of eicosapentaenoic acid per kg b.w., for the fish oil group.

From six hr after endotoxin through four days, there was better survival in the fish oil group ($p < .006$). Final mortality showed 7/11 fish-fed vs 2/11 safflower-fed animals surviving. We conclude that the administration of parenteral fish oil, even for a brief time, can have a profound effect on subsequent survival to endotoxin.
Lipids 23, 623–625 (1988).

Endotoxic shock involves activation of platelets and leukocytes (1). Investigations have shown enhanced survival to endotoxin when the effects of the vasoactive prostanoids thromboxane A_2 and prostacyclin I_2 have been inhibited through either decreased production or blocked action (2,3). Methods used to arrive at this common physiologic end-point include essential fatty acid deficiency, pretreatment with the cyclooxygenase inhibitors indomethacin or ibuprofen, or with the thromboxane synthetase inhibitor imidazole or by the use of 13-azaprostanoic acid, a thromboxane antagonist (2,3).

Fish oils contain eicosapentaenoic acid (EPA), a long chain polyunsaturated fatty acid of the ω3 family. As a substrate for cyclooxygenase in platelets, it forms thromboxane A_3, a vasoconstrictor like thromboxane A_2, but not a platelet aggregator (4,5). Likewise, in endothelial cells, EPA serves as substrate for prostacyclin I_3, which is similar to prostacyclin I_2 as a vasodilator and platelet anti-aggregator (5). This shifts the overall balance towards lessened clotting, manifested by prolonged bleeding times (6) and decreased thromboxane production (7).

Fish oils also have been shown to dampen leukocyte function (8). Lee and colleagues (8) showed decreased chemotaxis and decreased production of leukotriene B4 in neutrophils obtained from human subjects taking supplemental fish oil. This leukocyte inhibitory effect from dietary fish oil may be another mechanism through which survival to endotoxin challenge could be modulated.

We recently have shown (9) enhanced survival to an LD_{50} of endotoxin in animals orally fed fish oils for a six-wk period. We considered the hypothesis that fish oils would be protective against endotoxic shock by utilizing an IV preparation of fish oil given over several days.

METHODS

Animals. Male Hartley strain guinea pigs were obtained at a weight of 500 g from the Elm Hill Breeding Laboratories (Chelmsford, MA). They were housed in plastic cages with pine shavings on a 12-hr light and 12-hr dark cycle with the temperature controlled at 23 C and the relative humidity between 50–55%. This study met with the approval of the Institutional Animal Care and Use Committee of the New England Deaconess Hospital.

Study protocol. The animals were allowed a normal laboratory guinea pig diet (Agway Inc., Syracuse, NY), ad libitum, throughout the study. One wk after arriving, the animals underwent central vein catheterization. While anesthetized, a 0.025 in. (i.d.) × 0.037 in. (o.d.) silastic catheter was inserted into the superior vena cava from the internal jugular vein. The tubing was tunneled subcutaneously where it exited from the midscapular region and connected to a flow-through swivel that permitted free movement.

One day after the catheters were placed, the first of two days of lipid infusion was started. The animals received the respective lipid emulsions (safflower oil or fish oil) for two 24-hr periods separated by two days. Prior pilot studies revealed gross lipemia after one day of infusion, which cleared after two days of saline infusion. After the second day of lipid infusion, two more days were allowed to clear the second lipemia. On the morning of the seventh day, the animals received endotoxin intraperitoneally, 0.35 mg/100 g b.w., and then were followed for survival. Survival was noted at 3, 6, 9, 12, 15, 21, 24, 48, 72 and 96 hr after endotoxin injection. There were 11 animals in each group.

Emulsions. The emulsions used were each 10% emulsions. The fish oil emulsion was obtained from Baxter Healthcare Corp. (Deerfield, IL), and the safflower oil emulsion was from Abbott Laboratories (North Chicago, IL). Liposyn, the original formulation made solely from safflower oil and containing no soybean oil, was used. Table 1 depicts the fatty acid composition of the emulsions.

Endotoxin. Endotoxin was obtained from Difco Laboratories (Detroit, MI). The lipopolysaccharide was derived from *Escherichia coli* (026:B6, Lot #3920-10-9, Control #718687). Using the method of Reed and Muench (10), an LD_{50} of 0.35 mg/100 g b.w. was obtained for this lot of

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Abbreviation: EPA, eicosapentaenoic acid.

TABLE 1

Fatty Acid Composition of the IV Emulsions in Relative Percentages

	Fish oil	Safflower oil
Myristic (14:0)	8.1	
Palmitic (16:0)	12.5	7.0
Palmitoleic (16:1ω7)	7.3	
Stearic (18:0)	1.5	2.5
Oleic (18:1ω9)	6.1	13.9
Linoleic (18:2ω6)	5.9	77.0
α-Linolenic (18:3ω3)	1.2	0.1
Eicosapentaenoic (20:5ω3)	19.7	
Docosahexaenoic (22:6ω3)	8.6	

Fish oil emulsion made by Baxter Healthcare Corporation (Deerfield, IL). Safflower oil emulsion was Liposyn, made by Abbott Laboratories (North Chicago, IL) and contained no soybean oil.

TABLE 2

Lipid Intake

Parameter	Fish oil	Safflower oil
Body weight (b.w.)	500.8 ± 21.0	485.9 ± 24.3
Lipid infused (g/animal)	12.6 ± 0.3	13.0 ± 0.5
Lipid infused (g/kg b.w.)	25.4 ± 1.5	27.5 ± 2.5
Lipid infused (g/kg b.w./dose)	12.7 ± 0.1	13.7 ± 1.2
Lipid infused (g/kg b.w./hr)	0.53 ± 0.3	0.57 ± 0.05
EPA infused (gm/kg b.w.)	5.3 ± 0.3	0

Except for EPA, no weight or lipid differences by Student's t-test. N = 7 for each group. Data expressed as $\bar{x}$ ± SEM.

endotoxin in normal guinea pigs using the intraperitoneal route. The endotoxin was diluted in sterile saline to a maximum volume of 1 ml and injected intraperitoneally.

Anesthesia. A mixture of ketamine (44 mg/ml), acepromazine (0.2 mg/ml) and atropine (40 mcg/ml) was used for anesthesia at a dose of 1 ml/kg body weight, intramuscularly. Lidocaine as a 5% solution was used locally for any dissection.

Statistics. Survival was tested by Kaplan-Meier survival analysis using Surv-Pak-PC, software developed by the Johns Hopkins Oncology Center (Baltimore, MD).

RESULTS

Lipid intake. Table 2 shows the lipid intake for the two groups of animals. Each group received comparable amounts of fat, when analyzed by total amount over the two doses, amount per dose, per kilogram b.w. or rate of infusion. The total amount of EPA the fish oil-fed animals received over the six-day study was 5.3 ± .3 g/kg b.w.

Survival. Figure 1 shows the mortality among the two groups. As shown by Kaplan-Meier analysis, the two curves differ, $p < .006$. By nine hr after endotoxin administration, all nine safflower oil-fed animals that were to die had succumbed. In contrast, two of the four fish oil-fed animals that were to die lived for more than 21 hr.

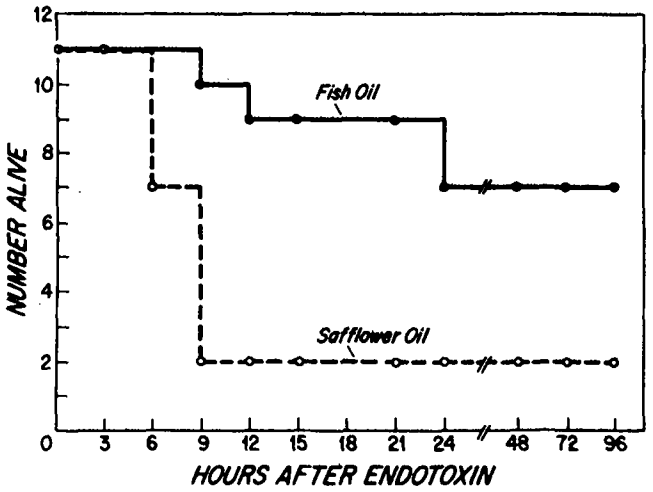

FIG. 1. Survival curves of the two dietary groups. Curves differ by Kaplan-Meier analysis, $p < 0.006$.

DISCUSSION

The beneficial impact of the fish oil may have been mediated through alteration in the metabolism of the prostanoids, thromboxane A_2 and prostacyclin I_2 from their respective tissues of origin, platelets and endothelium, or through leukotriene B_4 from leukocytes. The ability of EPA to antagonize the production of the more vasoconstrictor thromboxane A_2 or to diminish the generation of leukotriene B_4, thereby lessening leukocyte migration and endothelial adherence, could be its primary role. Alternatively, the presence of EPA-derived thromboxane A_3 and prostacyclin I_3 or leukotriene B_5 may have been involved. Because these compounds were not measured in this study, the temporal associations are not known. Further support for this explanation is data recently generated in guinea pigs fed a fish oil-based diet for six wk and then challenged with endotoxin (11). The metabolic acidosis that developed was much less pronounced as compared with those animals fed safflower oil.

Less linoleic acid and therefore less arachidonic acid as a substrate for eicosanoid formation may be another mechanism. This would view the fish oil as merely replacing an oil high in linoleic acid, i.e. safflower oil, leading to less arachidonic acid as substrate. Support for this comes from a recent study in which coconut oil-fed animals, as opposed to corn oil-fed animals, had better survival to endotoxin (12). Determining fatty acid profiles in plasma, platelets and leukocytes could resolve this question.

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REFERENCES

1. Goodman, M.L., Way, B.A., and Irwin, J.W. (1979) *J. Pathol. 128*, 7-14.

2. Cook, J.A., Wise, W.C., and Halushka, P.V. (1980) *J. Clin. Invest. 65*, 227–230.
3. Wise, W.C., Cook, J.A., Eller, T., and Halushka, P.V. (1980) *J. Pharmacol. Exp. Ther. 215*, 160–164.
4. Fischer, S., and Weber, P.C. (1983) *Biochem. Biophys. Res. Commun. 116*, 1091–1099.
5. Needleman, P., Raz, A., Minkes, M.S., Ferrendelli, J.A., and Sprecher, H. (1979) *Proc. Natl. Acad. Sci. USA 76*, 944–48.
6. Goodnight, S.H., Harris, W.S., and Connor, W.E. (1981) *Blood 58*, 880–885.
7. Hornstra, G., Christ-Hazelhof, E., Haddeman, E., TenHoor, F., and Nugteren, D.H. (1981) *Prostaglandins 21*, 727–739.
8. Lee, T.H., Hoover, R.L., Williams, J.D., Sperling, R.I., Ravalese, J. III, Spur, B.W., Robinson, D.R., Corey, E.J., Lewis, R.A., and Austin, K.F. (1985) *N. Engl. J. Med. 312*, 1217–1224.
9. Mascioli, E.A., Iwasa, Y., Trimbo, S., Leader, L., Bistrian, B.R., and Blackburn, G.L., *Am. J. Clin. Nutr.*, in press.
10. Reed, L.J., and Muench, H. (1938) *Am. J. Hygiene 27*, 493–497.
11. Pomposelli, J., Bistrian, B.R., Blackburn, G.L., and Zeisel, S. (1988) *JPEN 12*, 9S.
12. Wan, J.M., and Grimble, R.F. (1987) *Clin. Sci. 72*, 383–385.

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Identification of N-ε-(2-propenal)lysine as a Major Urinary Metabolite of Malondialdehyde

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N-ε-(2-propenal)lysine (ε-PL) was identified as one of two major metabolites of malondialdehyde (MDA) excreted in rat and human urine. This compound is derived mainly but not exclusively from the diet, where it arises from a reaction between free MDA generated in the oxidative decomposition of polyunsaturated fatty acids and the ε-amino of the lysine residues of food proteins. It is released during protein digestion and represents the main form in which MDA is absorbed. It is excreted partially in unchanged form and partially as the acetylated derivative N-α-acetyl-N-ε-(2-propenal)lysine. Its administration to rats did not result in an increase in the excretion of free MDA in the urine. The findings that MDA in foods is absorbed mainly as ε-PL, and that this compound is not metabolized to free MDA in vivo, mitigate concern over the possible mutagenicity and carcinogenicity of MDA in the diet.
Lipids 23, 626–628 (1988).

Work in this laboratory has revealed the presence of several malondialdehyde (MDA) derivatives in rat and human urine (1–3). Figure 1 illustrates an elution profile of MDA compounds obtained by anion exchange column chromatography of rat urine. One of two major metabolites (Fig. 1F) has been identified as N-α-acetyl-N-ε-(2-propenal)lysine (1) and two minor metabolites as MDA adducts with the phospholipid bases ethanolamine (Fig. 1B) and serine (Fig. 1E) (2,3). Free MDA (Fig. 1G) was detected in some urine samples. This paper describes the identification of the second major urinary metabolite of MDA (Fig. 1A) as the unacetylated lysine-MDA adduct N-ε-(2-propenal)lysine.

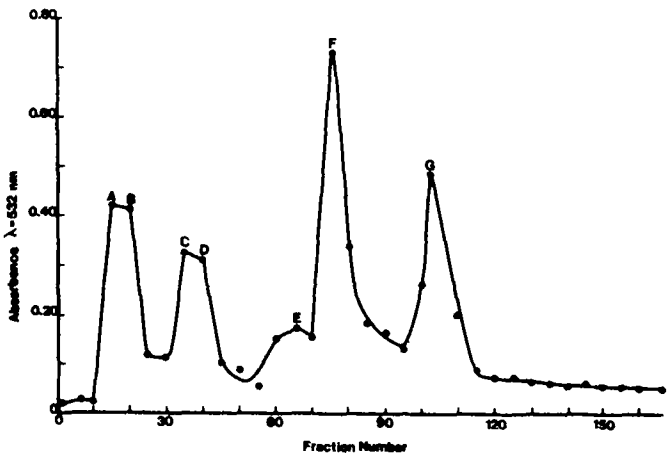

FIG. 1. Elution profile of MDA derivatives obtained by anion exchange chromatography of urine from rats fed a vitamin E deficient diet containing cod liver oil (2). A, N-ε-(2-propenal)lysine; B, N-(2-propenal) ethanolamine; E, N-(2-propenal)serine; F, N-α-acetyl-N-ε-(2-propenal)lysine; G, free MDA. C and D, unidentified. MDA was determined spectrophotometrically as the thiobarbituric acid derivative (9). Reproduced with permission (2).

METHODS

The procedure used for the isolation of urinary metabolites of MDA has been described in detail elsewhere (1). Briefly, it includes anion exchange, cation exchange and size exclusion column chromatography, followed by high performance liquid chromatography (HPLC) on preparative and analytical reverse phase columns.

The occurrence of an acetylated lysine-MDA adduct in urine (1) suggested the co-existence of the unacetylated adduct N-ε-(2-propenal)lysine. This compound (ε-PL) therefore was synthesized by a modification (1) of the procedure of Nair et al. (4). This procedure yields a mixture of N-α and N-ε adducts (α-PL and ε-PL) (Fig. 2). Synthetic ε-PL was subjected to the chromatographic procedures used for the isolation of urinary metabolites, and its elution pattern was compared with those of the remaining unidentified compounds (Fig. 1).

RESULTS

When subjected to anion exchange column chromatography, synthetic ε-PL eluted in the same fractions as a major urinary metabolite of MDA (Fig. 1A). However,

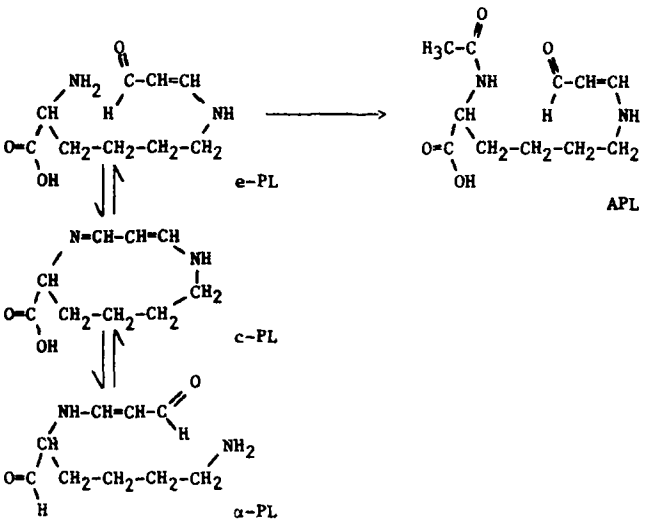

FIG. 2. Interconversions of lysine-MDA adducts. APL is a major form of MDA found only in urine (1). ε-PL is the main form of MDA absorbed from the diet (8) and is also a major form in urine. c-PL and α-PL are formed from ε-PL as isolation artifacts.

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Abbreviations: α-PL, N-α-acetyl-N-ε-(2-propenal)lysine; c-PL, cyclized product; ε-PL, N-ε-(2-propenal)lysine; MDA, malondialdehyde; HPLC, high pressure liquid chromatography.

when subjected to analytical reverse phase HPLC, authentic ε-PL and the purified metabolite failed to co-elute.

Coincidentally, storage of pure synthetic ε-PL in aqueous solution at 4 C was observed to result in its partial conversion to two additional compounds (Fig. 3). One of these compounds was found to co-elute with pure, synthetic α-PL when chromatographed on a reverse phase HPLC column. The other co-chromatographed with the MDA derivative isolated from urine (Fig. 4). Under similar storage conditions, α-PL was partially converted to ε-PL and the unidentified product of ε-PL conversion. It was apparent from these observations that the unidentified compound was an intermediate in the interconversion of α-PL and ε-PL.

Characterization of the intermediate by NMR and IR was confounded by its retroconversion to α-PL and ε-PL. Its UV_{max} at 280 nm and its fluorescence spectrum indicated that the Schiff's base bonds present in α-PL and ε-PL were intact. In aqueous solution, it exhibited fluorescence (Ex_{max} 370 nm, Em_{max} 467 nm) consistent with the presence of the general structure R—NH—CH=CH—CH=N—R (5). Cation exchange chromatography showed that it was less positively charged than α-PL and ε-PL. On the basis of these observations, it was concluded that the compound was a cyclized product (c-PL) of intramolecular reactions between the free aldehyde and amino groups of α-PL and ε-PL (Fig. 2).

Anion exchange chromatography of pure, synthetic ε-PL resulted in its almost complete conversion to the cyclized form (Fig. 5), indicating that the c-PL recovered from urine was formed from ε-PL as an isolation artifact. No evidence was obtained for the natural occurrence of either c-PL or α-PL in urine, although their presence in small amounts cannot be excluded.

DISCUSSION

N-ε-(2-propenal)lysine and its N-α-acetylated derivative are the main forms in which MDA is excreted in rat and human urine. Under normal conditions, both compounds are mainly of dietary origin. ε-PL appears to be formed by a reaction between free MDA generated in the oxidative decomposition of polyunsaturated fatty acids in the diet and the ε-NH_2 groups of lysine residues of food proteins, from which it is released during protein digestion.

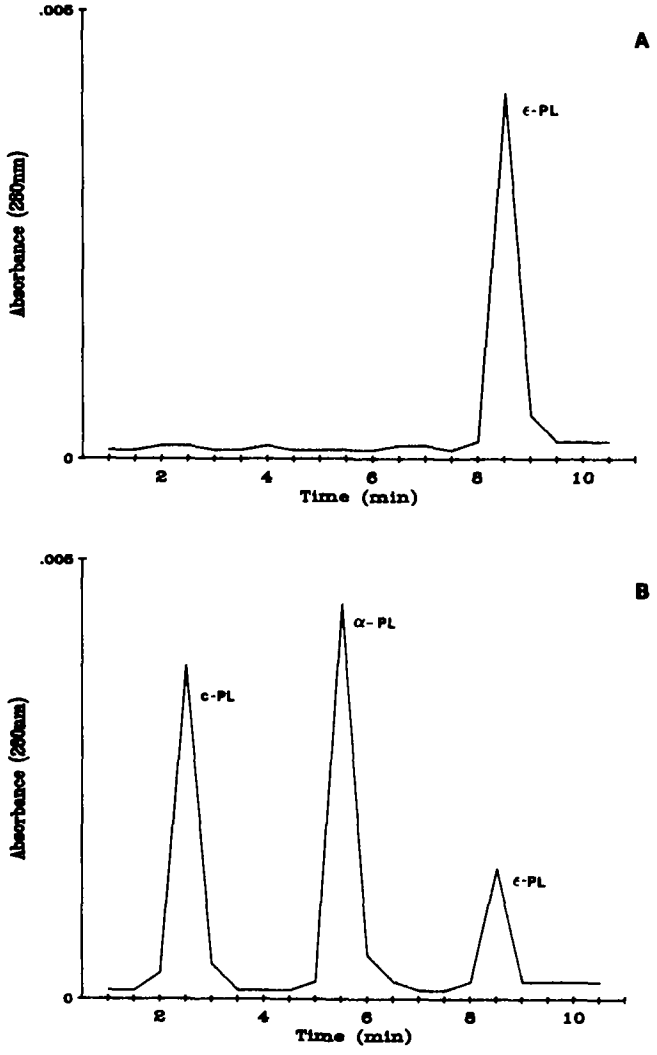

FIG. 3. Reverse phase HPLC elution profile of synthetic ε-PL before (A) and after (B) storage in aqueous solution at 4 C for several weeks.

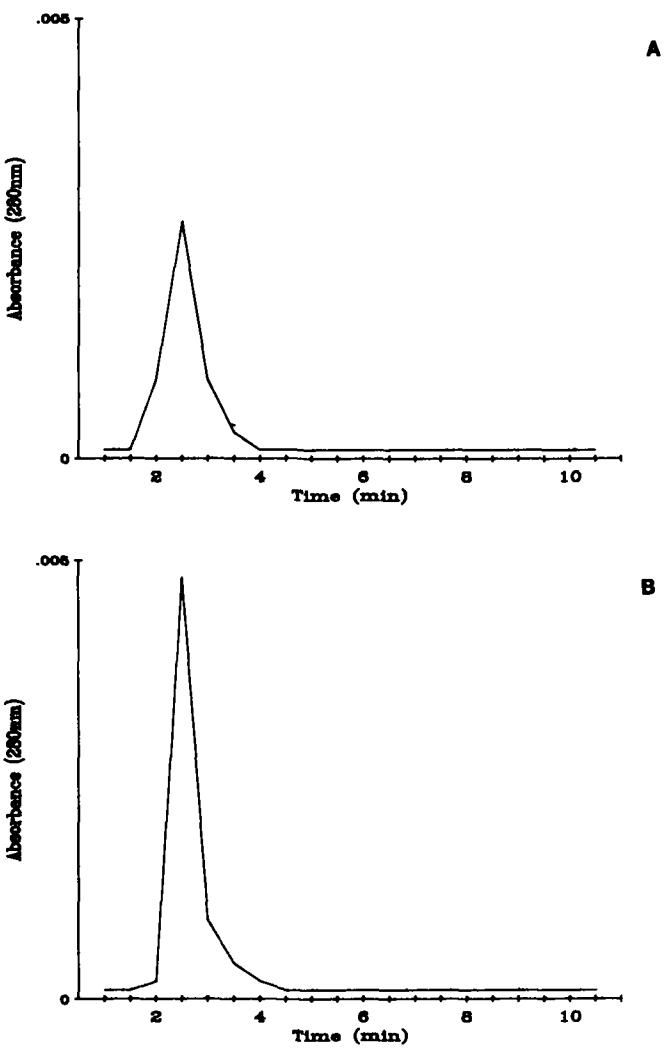

FIG. 4. Reverse phase HPLC elution profile of urinary MDA isolate obtained before (A) and after (B) spiking with synthetic c-PL.

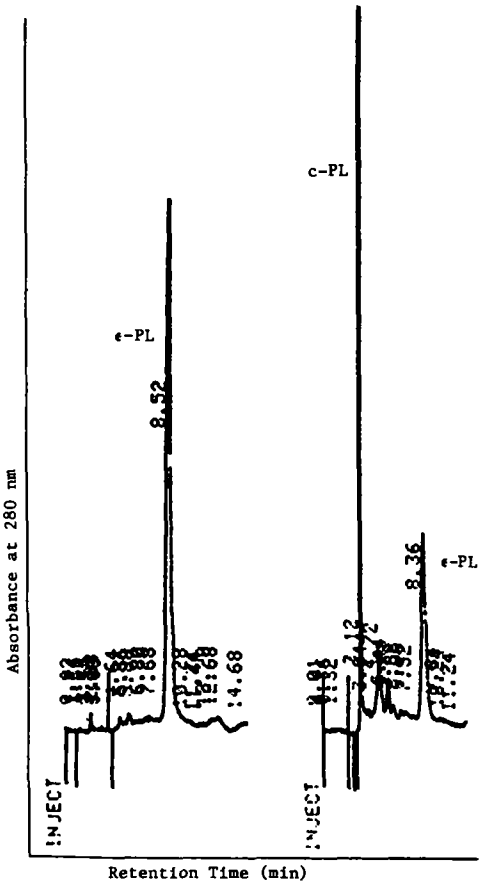

FIG. 5. Reverse phase HPLC elution profile of ε-PL before (A) and after (B) chromatography on anion exchange resin.

Chromatographic fractionation of the in vitro digestion products of several foods showed that ε-PL was the predominant form of MDA present (6). It is excreted partially in the acetylated form. The liver is the main site of N-acetylation reactions.

Intraperitoneal administration to rats of 60 μg of MDA as ε-PL produced no increase in the excretion of free MDA in the urine. Because the excretion of free MDA is responsive to smaller doses of MDA as the sodium enol salt (1), it appears that ε-PL is not a source of free MDA in vivo. The findings that ε-PL is the main form of MDA absorbed from the diet (6) and that this compound is not metabolized to free MDA in the body mitigate concern over the possible mutagenicity (7) and carcinogenicity (8) of dietary MDA.

Although the excretion of lysine-MDA adducts by the rat is markedly decreased by fasting or feeding a diet containing only saturated fat (hydrogenated coconut oil), these compounds are nevertheless detectable in urine, indicating that they are formed in the tissues as well as in the diet. They also are present in human urine collected 12–36 hr after the beginning of a fast, and collected while consuming a protein-free diet. Hence, they appear to reflect reactions of MDA with cellular as well as dietary proteins.

ACKNOWLEDGMENT

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REFERENCES

1. McGirr, L.G., Hadley, M., and Draper, H.H. (1985) *J. Biol. Chem. 260,* 15427–15431.
2. Hadley, M., and Draper, H.H. (1988) *Fed. J.,* 138–140.
3. Hadley, M., and Draper, H.H., *Free Radicals in Biol. and Med.,* in press.
4. Nair, V., Vietti, D.E., and Cooper, C.S. (1981) *J. Am. Chem. Soc. 103,* 3030–3036.
5. Chio, K.S., and Tappel, A.L. (1969) *Biochemistry 8,* 2821–2827.
6. Piché, L.A., Cole, P.D., Hadley, M., van den Bergh, R., and Draper, H.H., *Carcinogenesis,* in press.
7. Basu, A.K., and Marnett, L.J. (1983) *Carcinogenesis (Lond.) 4,* 331–333.
8. Shamberger, R.J., Andreone, T.L., and Willis, C.E. (1974) *J. Natl. Cancer Inst. 53,* 1771–1773.
9. Shinnhuber, R.O., and Yu, T.C. (1958) *Food Technol. 12,* 9–12.

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Evidence for Loss of Tritium from 3β-Tritiated Deoxycholic Acid during Enterohepatic Cycling in Man

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A double-label single-intubation isotope technique was assessed for studying deoxycholic acid metabolism by simultaneous IV administration of [3β-^{3}H]deoxycholic acid and [24-^{14}C]deoxycholic acid to five healthy subjects. The ^{3}H/^{14}C ratio in duodenal bile fell progressively in all subjects, the mean reduction on day four being 48.8%. When the same labeled bile acids were administered to three ileostomy subjects, again the ratio fell with a mean reduction of 44.4% on day four. This fall more likely was due to hepatic exchange of tritium for hydrogen, rather than to colonic bacterial oxidation. [3β-^{3}H]Deoxycholic acid should not be used to study the kinetics and metabolism of deoxycholic acid.
Lipids 23, 629–630 (1988).

Vantrappen et al. (1) recently described an isotopic technique for determining the turnover rate of cholic acid from a single sample of bile, which they compared with four- or five-day samples as required by the classical Lindstedt method (2). This new technique requires administration of ^{3}H-labeled as well as of ^{14}C-labeled cholic acid. We intended to study deoxycholic acid pool size and kinetics in relation to colonic transit (3,4) and, attracted by the simplicity of the Vantrappen technique, decided to investigate its feasibility with deoxycholic acid. Because tritiated deoxycholic acid is not commercially available, Amersham International PLC was commissioned to synthesize it for us, the ^{3}H-label being inserted in the 3β-position on the bile acid nucleus. It is recognized that some tritiated bile acids are unsuitable as markers because ^{3}H is exchanged for hydrogen in body water (5–7). Recently, it has been suggested that oxido-reduction of the hydroxyl group at the 3- and 12-position of deoxycholic acid by the intestinal microflora can occur (8). Thus, both mechanisms could result in a fall in specific radioactivity not due to turnover of the bile acid. At the time of study, bile acids tritiated in the 3β position had not been assessed for stability. To examine this, we measured the ratio of ^{3}H/^{14}C radioactivity in biliary deoxycholic acid during enterohepatic circulation after the deoxycholic acid pool had been labeled simultaneously with tracer amounts of [3β-^{3}H]deoxycholic acid and [24-^{14}C]deoxycholic acid. If [3β-^{3}H]deoxycholic acid is stable during enterohepatic circulation, then the ^{3}H/^{14}C ratio should remain constant.

MATERIALS AND METHODS

[3β-^{3}H]Deoxycholic acid was prepared by the selective oxidation of the 3-position of methyl deoxycholate with silver carbonate on celite. The methyl-3-oxo-12α-hydroxy-5β-cholan-24-oate oxidation product was purified by column chromatography and then reduced by sodium boro-[^{3}H]-hydride to the 3α-hydroxyl epimer, which subsequently was purified by thin layer chromatography (TLC). The methyl ester was saponified with sodium hydroxide. Radiochemical purity by TLC was 98%, and the specific activity was 21.2 C/mmol.

Approximately 10 μC of [24-^{14}C]deoxycholic acid and 30 μC of [3β-^{3}H]deoxycholic acid were injected intravenously into four healthy female and one healthy male volunteer, mean age 49 years (range 42–64), three hr after their last meal of the day. The ^{3}H and ^{14}C radioactivities were measured in small aliquots of thoroughly mixed duodenal bile samples collected after cholecystokinin injection on the next four mornings, according to the Lindstedt method (2). In view of the results obtained in these five subjects, the procedure was repeated in three more subjects (two females and one male, mean age 43 years; range 36–51), who had undergone total colectomy and ileostomy for ulcerative collitis at least five years before. In these subjects, bile samples were collected only on the first and fourth morning after their injection. All volunteers gave their informed consent for the study, which was approved by the hospital ethical committee.

After precipitation of bile proteins by boiling methanol, the bile acids were deconjugated with cholylglycine hydrolase (9), extracted in ether and separated by TLC using as development solvent 2,2,4-trimethylpentane/ethyl acetate/glacial acetic acid (5:5:1, v/v/v) (10). The dihydroxy bile acids were eluted and assayed for ^{3}H and ^{14}C radioactivity in a dual-channel liquid-scintillation counter with quench correction by external standardization and the ratio of ^{3}H/^{14}C was calculated.

RESULTS AND DISCUSSION

In the five normal subjects, the ^{3}H/^{14}C ratio fell progressively, with a mean reduction of 48.8% by the fourth day (range 41.5–57.1%) (Fig. 1). In the three ileostomy subjects, the ^{3}H/^{14}C ratio fell by a mean of 44.4% by the fourth day (71.3, 31.5 and 26.9%).

The results in the five healthy volunteers indicate that [3β-^{3}H]deoxycholic acid loses radioactivity during enterohepatic cycling. After this study was performed, Björkhem et al. (8) published similar findings for healthy subjects. We considered two causes for our results: exchange of tritium for hydrogen within the liver (11) and loss of tritium as a result of 3-oxidation by the colonic bacterial flora followed by reduction either in the colon or liver with incorporation of a nonradioactive hydrogen atom and circulation of the now unlabeled bile acid.

Subjects with ileostomies have no or very small amounts of deoxycholic acid in the bile (12,13) because of the relative paucity of anaerobic bacteria able to dehydroxylate cholic acid (14). If bacterial oxidation and reduction of [3β-^{3}H]deoxycholic acid had contributed to the fall of the ^{3}H/^{14}C ratio in the normal subjects, then in the ileostomy patients the ratio should have remained constant or fallen much less, which was not the case. Our

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Abbreviation: TLC, thin layer chromatography.

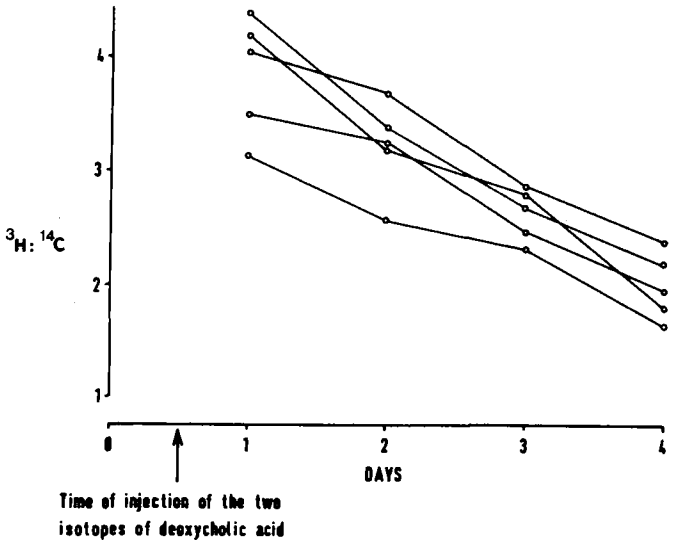

FIG. 1. Ratio of $^3H/^{14}C$ in duodenal bile samples collected on four consecutive mornings from five healthy volunteers following the simultaneous IV administration of tracer amounts of [3β-^{3}H]deoxycholic acid and [24-^{14}C]deoxycholic acid.

results in ileostomy subjects contrast with those of Björkhem et al. (8). But their finding of a slower decline in the $^3H/^{14}C$ ratio in two ileostomy subjects than in seven healthy controls was made after the administration of [12β-^{3}H]deoxycholic acid and not [3β-^{3}H]deoxycholic acid. This indicates that the loss of label in the 12β-position is due mainly to bacterial oxidation. However, our results suggest the same mechanism cannot be assumed to apply to [3β-^{3}H]deoxycholic acid.

We conclude that bacterial oxido-reduction of deoxycholic acid at the 3-position is unlikely to occur to any great extent during its enterohepatic cycling in man, and that loss of the label is more likely due to hepatic exchange of tritium with hydrogen (11). Studies in subjects whose bile flow has been diverted away from the colon, such as in post-cholecystectomy T-tube patients, would help confirm these findings.

When considering the use of tritiated bile acids, recommendations of Panveliwalla et al. (7) should be remembered. The data from our study confirm that [3β-^{3}H]deoxycholic acid loses tritium during enterohepatic cycling, and that it should not be used to measure deoxycholic acid pool size or kinetics. If [2-4-^{3}H]deoxycholic acid (analogous to [2-4-^{3}H]cholic acid used in the original Vantrappen technique) had been chosen instead, the results would probably have been similar, because it also loses label (7). Instead, [22,23-^{3}H]deoxycholic acid could be tried; however, it is not commercially available and would need to be specially synthesized and then tested for its suitability as a label.

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REFERENCES

1. Vantrappen, G., Rutgeerts, P., and Ghoos, Y. (1981) *J. Lipid Res. 22,* 528-531.
2. Lindstedt, S. (1957) *Acta Physiol. Scand. 40,* 1-9.
3. Marcus, S.N., and Heaton, K.W. (1986) *Gut 27,* 550-558.
4. Marcus, S.N., and Heaton, K.W. (1986) *Gut 27,* 893-900.
5. LaRusso, N.F., Hoffman, N.E., and Hofmann, A.F. (1974) *J. Lab. Clin. Med. 84,* 759-765.
6. Einarsson, K., Hellström, K., and Kallner, M. (1974) *Clin. Chim. Acta 56,* 235-239.
7. Panveliwalla, D.K., Pertsemlidis, D., and Ahrens, E.H. (1974) *J. Lipid Res. 15,* 530-532.
8. Björkhem, I., Liljeqvist, L., Nilsell, K., and Einarsson, K. (1986) *J. Lipid Res. 27,* 177-182.
9. Nair, P.P., Gordon, M., and Reback, J. (1967) *J. Biol. Chem. 242,* 7-11.
10. Thornton, J.R., Emmett, P.M., and Heaton, K.W. (1983) *Gut 24,* 2-6.
11. Cronholm, T., Makino, I., and Sjövall, J. (1972) *Eur. J. Biochem. 26,* 251-258.
12. Percy-Robb, I.W., Jalan, K.N., McManus, J.P.A., Gould, J.C., and Sircus, W. (1971) *Clin. Sci. 41,* 321-382.
13. Morris, J.S., Low-Beer, T.S., and Heaton, K.W. (1973) *Scand. J. Gastroenterol. 8,* 425-431.
14. Gorbach, S.L., Nahas, L., Weinstein, L., Levitan, R., and Patterson, J.F. (1957) *Gastroenterology 53,* 874-880.

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ERRATUM

Several lines of the text in "Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: X. Volatile Thermal Decomposition Products of Methyl Linolenate Dimers" by E. N. Frankel et al. (*Lipids 23*, 295–298) were inadvertently deleted. At the bottom of the first column of page 295, there should be the phrase ". . . contained hydroperoxy epidioxides, dihydroperoxides and monohydroperoxides joined with peroxide and ether/. . . ."

Comparison of Fatty Acid and Triacylglycerol Metabolism of Macrophages and Smooth Muscle Cells

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The response of macrophages and smooth muscle cells to culture in free fatty acid has been compared. Because oleate and linoleate promoted triacylglycerol enrichment of smooth muscle cells, whereas palmitate had little effect, oleate was used for these studies. The kinetics of the accumulation of triacylglycerol produced by oleate was comparable between smooth muscle cells and macrophages. When grown in increasing concentrations of oleic acid at various fatty acid to albumin molar ratios, the extent of triacylglycerol accumulation in both cell types was dependent on the concentration of oleate, the concentration of albumin, and the oleate to albumin molar ratio. However, macrophages contained 2.6-fold more triacylglycerol than smooth muscle cells in the presence of oleate at 0.36 mM or greater and at levels of albumin higher than 0.15 mM. The cellular triacylglycerol content of macrophages was linearly related to the oleate to albumin molar ratio at both a constant albumin concentration and a constant oleate concentration, whereas the accumulation of triacylglycerol in smooth muscle cells showed a curvilinear relationship. When cells were preloaded with triacylglycerol, smooth muscle cells showed a greater loss of lipid when exposed to albumin than macrophages did. Over a two-hr time period, macrophages incorporated twice as much labeled fatty acid as smooth muscle cells. Thus, while smooth muscle cells and macrophages showed similar responses to exogenous fatty acid and albumin, there were also significant quantitative distinctions.

Lipids 23, 641–646 (1988).

One of the principal sources of lipid for cells grown in tissue culture is fatty acids derived from the serum in the medium (1). Most of the plasma fatty acids are esterified in the form of triacylglycerol, phospholipids and cholesteryl esters, with a small portion found as free fatty acid bound to albumin. Due to their high turnover rate, free fatty acids provide a significant portion of the lipid requirements of tissues (2,3). Cells in culture readily take up free fatty acids from the medium via an energy-independent process. Once the fatty acids are incorporated into the cell, they are either oxidized or esterified (4). Free fatty acid transport within the cell probably is mediated by the long chain fatty acid-binding "Z" protein (5,6).

The two primary sources of fatty acids for the arterial wall cells include the free fatty acids present as such in the plasma and the free fatty acids that result from the hydrolysis of chylomicrons and very low density lipoproteins (VLDL) through the action of lipoprotein lipase either on the luminal face of the blood vessel or from resident macrophages. Several past and recent observations provide suggestive evidence that these may be elevated or altered in atherosclerosis. A) Numerous investigations have demonstrated either an elevation in plasma fatty acid concentration or an increase in the plasma free fatty acid to albumin molar ratio in patients who have coronary heart disease or who demonstrate several of the risk factors associated with the disease such as diabetes mellitus, smoking, aging and stress (7–11). B) The endothelium of the diseased vessel wall is more permeable to albumin, the carrier of fatty acids (12,13). C) Atherosclerotic aortas demonstrate an increase in lipoprotein lipase activity that would increase the arterial free fatty acid content (14). Macrophages, found in greater numbers in plaques (15, 16), also may contribute to the fatty acid pool of the vessel wall because they have been shown to secrete lipoprotein lipase (17). D) Finally, in our previous studies we found that the metabolism of normal triacylglycerol-rich VLDL by macrophages, in the presence of albumin, resulted in an enrichment of the medium with free fatty acids (18). We have shown that these fatty acids produced triacylglycerol accumulation in smooth muscle cells (19), indicating that the interaction of these two cell types in the diseased blood vessel wall may be a significant determinant in local lipid accumulation. Because smooth muscle cells and macrophages are the principal cell types of the atherosclerotic lesion, their response to exogenous free fatty acids and the resultant triglyceride accumulation may prove important in the disease process, particularly in view of the report of Adelman et al. (20) that the triglyceride content of cells influences their ability to clear intracellular cholesteryl esters.

Smooth muscle cells and macrophages differ greatly in their metabolism of lipids. Smooth muscle cells obtain cholesterol from low density lipoproteins (LDL). LDL uptake is mediated by cell surface receptors that also will bind VLDL (21–23). In contrast, macrophages probably have few LDL receptors, but contain receptors for triacylglycerol-rich VLDL and cholesteryl ester-rich, beta-migrating VLDL (β-VLDL). Although smooth muscle cells will accumulate some cholesteryl esters when grown with LDL isolated from hyperlipemic animals, these levels are not comparable with the massive levels of cholesteryl esters found in macrophages exposed to β-VLDL from animals on a high-fat, high-cholesterol diet (24). In addition, macrophages store large amounts of triacylglycerol when incubated with normal triacylglycerol-rich VLDL, whereas smooth muscle cells show only slight changes in triacylglycerol content (18). Thus, it was expected that the two cell types would show differences in their metabolism of fatty acids. This study examines the response of these two cell types to exogenous free fatty acid and reports on the differences in their capacity for triacylglycerol production, accumulation and turnover.

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Abbreviations: BME, Basal Medium of Eagle; BSA, bovine serum albumin; B-VLDL, beta-migrating very low density lipoprotein; CE, cholesteryl ester; DMEM, Dulbecco's Modified Eagle's Medium; FA, fatty acid; FFA, free fatty acid; J774, macrophages; LDL, low density lipoproteins; PBS, phosphate-buffered saline; PL, phospholipid; SMC, smooth muscle cells; TG, triacylglycerol; VLDL, very low density lipoproteins.

MATERIALS AND METHODS

Tissue culture. Arterial medial smooth muscle cells were obtained from outgrowths of explants from the thoracic aorta of Macaca mulatta monkeys (rhesus) as described (25,26), grown in Basal Medium of Eagle (BME) with 5% bovine calf serum (K.C. Biological, Lenaxa, KS), and used between the third and sixth passages (0.2–1.0 mg protein/flask). Experiments were carried out in 25 cm^2 flasks (Falcon) in a volume of two ml per flask.

The macrophage-like cell line J774 A.1 (J774) (27) was purchased from the American-Type Culture Collection and was maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (K.C. Biological). Experiments were performed in Multiwell tissue culture plates (12 wells/plate, 0.2–0.6 mg protein/well) at a volume of 1 ml per well. J774 cells were chosen as a model for macrophages because their lipid metabolism has been shown to resemble that of several types of macrophages. For example, mouse peritoneal macrophages and J774 cells secrete lipoprotein lipase (18,28), show similar rates of cholesterol esterification upon exposure to β-VLDL (29), accumulate comparable amounts of triacylglycerol upon exposure to normal VLDL (18) or oleate (30), and demonstrate the same rate of release of labeled free fatty acid (30). Unlike mouse peritoneal macrophages, J774 cells have LDL receptors and, in this regard, they resemble monocyte macrophages (31).

At the start of an experiment, cells were washed twice with sterile phosphate-buffered saline (PBS). One or 2 ml of test medium consisting of DMEM or BME plus additions was added, and the cells were incubated for the appropriate time periods in a 5% CO_2 atmosphere at 37 C. To terminate an experiment, cells were washed three times with PBS and removed from the well by exposure to 0.1 N NaOH for 20 min at room temperature. This brief treatment with NaOH does not hydrolyze intracellular triglycerides or cholesteryl esters. Those dissolved cells sampled for extraction were immediately neutralized with concentrated acetic acid.

Fatty acids and radiolabeled compounds. Fatty acid-free fraction V bovine serum albumin (BSA) was purchased from Miles Labs (Elkhart, IN). Lipids were procured as follows: oleic acid (18:1) and palmitic acid (16:0) from Sigma Chemical Co. (St. Louis, MO), linoleic acid (18:2) from Supelco (Bellefont, PA), [1-C^{14}] oleic acid (57 Ci/mol) from Amersham/Searle (Arlington Heights, IL), [4-^{14}C]cholesterol (54 Ci/mol) and [1,2 − ^{3}H (N)] cholesterol (54.8 Ci/mol) from New England Nuclear (Boston, MA).

Fatty acids were complexed to BSA according to the procedure of St. Clair et al. (32). Fatty acids dissolved in 95% ethanol and dried down under N_2, were bound to 17% BSA in phosphate-buffered saline at 37 C at various molar ratios and concentrations. These solutions were sterilized by filtration through a 0.45 μm Miller-HA filter (Millipore). The experiments were performed using 25, 50, 100, 200 and 300 μg/ml oleate or 0.09, 0.18, 0.36, 0.71 and 1.07 mM oleate.

Analysis. Cells and media were extracted using the method of Bligh and Dyer (33). The triacylglycerol and free fatty acid content of the cells and media were quantitated using the method of Marsh and Weinstein (34) as modified by Kritchevsky et al. (35). Briefly, the extracted lipids were separated on Silica Gel G glass thin layer chromatography plates (Fisher Scientific, Pittsburgh, PA), developed with petroleum ether/ethyl ether/acetic acid (75:25:1, v/v/v), scraped, charred with sulfuric acid, and analyzed spectrophotometrically. [4-^{14}C]Cholesterol added during the extraction served as an internal standard, and results were corrected for recovery (mean recovery = 80%).

[4-^{14}C]Oleate incorporation into cholesteryl esters, triacylglycerol, fatty acids and phospholipids were measured by separating the lipids on silica gel IB2 plastic thin layer chromatography sheets (Baker, Phillipsburgh, NJ). The sheets were then cut and counted. [1,2 − ^{3}H (N)] cholesterol served as an internal standard (26). Protein was determined by the method of Lowry et al. (36), and triacylglycerol content of cells is expressed as n mol/mg cell protein based on the molecular weight of glycerol trioleate (mw. 886).

RESULTS

In recent studies, we have shown that free fatty acids will promote triacylglycerol accumulation in macrophages and smooth muscle cells (18,19) as had been demonstrated in other cell types (37,38). To determine whether different fatty acids would cause variations in intracellular triacylglycerol levels, the ability of oleic acid (18:1) to affect the triacylglycerol content of smooth muscle cells was compared with that of linoleate (18:2) and the saturated fatty acid palmitate (16:0) at two different fatty acid to albumin molar ratios. As seen in Figure 1, both oleate and linoleate promoted triacylglycerol enrichment of the smooth muscle cells, although oleate was more effective. In contrast, palmitate had little effect on the triacylglycerol content of the smooth muscle cells at either fatty acid to albumin molar ratio. Thus, oleate was used for subsequent experiments.

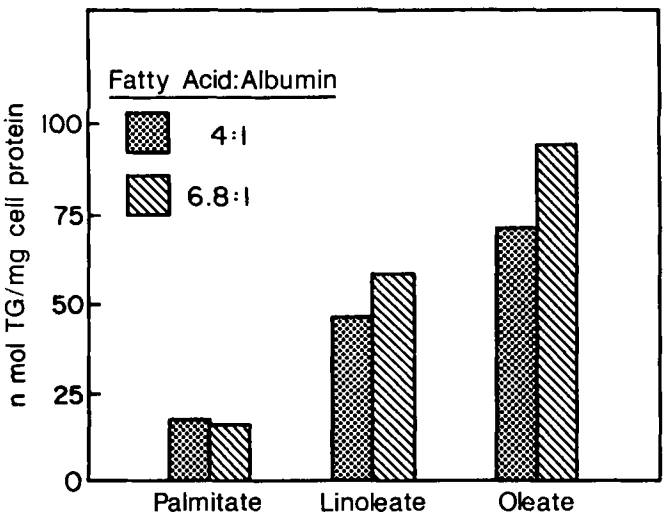

FIG. 1. The influence of various fatty acids on the triacylglycerol content of smooth muscle cells. Smooth muscle cells were incubated for 24 hr with 200 μg/ml of palmitate (0.78 mM), linoleate (0.71 mM) or oleate (0.71 mM), at two different fatty acid to albumin molar ratios. Data are the average of duplicate determinations. TG, triacylglycerol.

A comparison of the increment in cellular triacylglycerol content of monkey arterial smooth muscle cells and J774 macrophages exposed to 0.71 mM oleate for 24 hr at a fatty acid to albumin molar ratio of 4:1 is illustrated in Figure 2. Incubation in oleate stimulated the formation of oil red 0 positive droplets in both cell types (data not shown). Under these conditions, the final intracellular triacylglycerol levels were greater in the J774 macrophages. However, when the two cell types were exposed to the same oleate concentration (0.71 mM oleate) but at a higher oleate to albumin molar ratio (6.8:1, oleate/albumin), the extent of triacylglycerol accumulation after 24 hr was similar in the smooth muscle cells and macrophages. To summarize several experiments performed under these latter conditions, smooth muscle cells contained 166 ± 41 n mol triacylglycerol/mg cell protein (x ± S.D., n = 14) after 24 hr incubation with oleate (6.8:1, oleate/albumin) and macrophages contained 194 ± 28 n mol triacylglycerol/mg cell protein (x ± S.D., n = 8).

Figure 3 illustrates that the kinetics of enrichment in triacylglycerol mass was similar between smooth muscle cells and macrophages. There was an initial lag period of two to four hr for the smooth muscle cells or a slow accumulation in macrophages, followed by a rapid increase in triacylglycerol levels in both cell types up to 8 to 10 hr; this was followed by a slower but continuous rise in lipid up to 24 hr of incubation.

To characterize the difference in the oleate-stimulated enrichment of cellular triacylglycerol between macrophages and smooth muscle cells in greater detail, the two cell types were grown in increasing concentrations of oleic acid at various fatty acid to albumin molar ratios. As shown in Figures 4 and 5, the extent of triacylglycerol accumulation in both cells was dependent on the concentration of albumin and the oleate to albumin molar ratio.

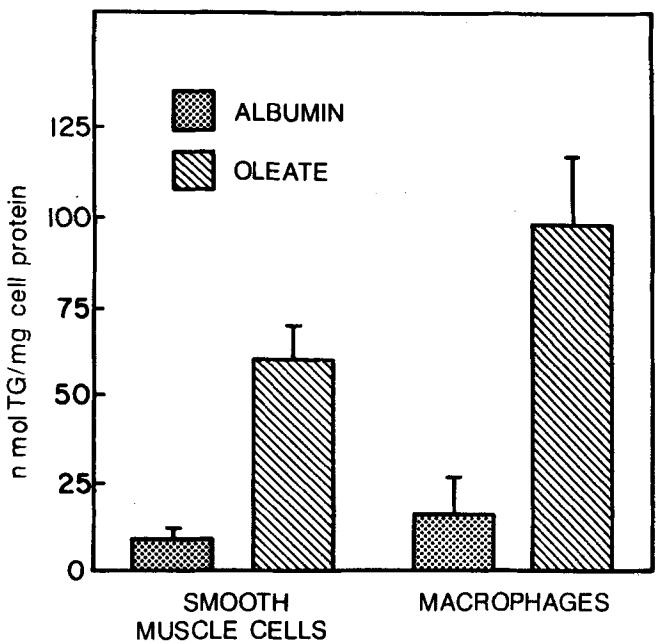

FIG. 2. Stimulation of intracellular triacylglycerol accumulation by oleate. Smooth muscle cells and J774 macrophages were incubated for 24 hr with oleate/albumin at a molar ratio of 4:1 and a concentration of 0.71 mM oleate or with albumin (0.15 mM). x ± S.D. (n = 6); TG, triacylglycerol.

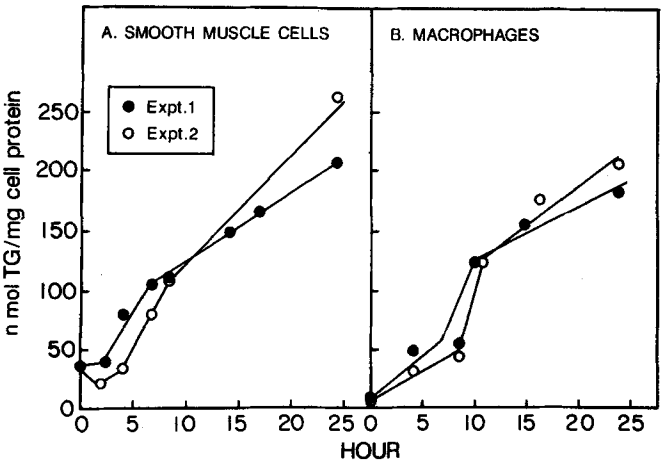

FIG. 3. Comparison of time course of triacylglycerol enrichment between smooth muscle cells and macrophages. Smooth muscle cells or J774 macrophages were incubated with 0.71 mM oleate at an oleate/albumin ratio of 6.8 to 1 for the indicated time period. The values are the average of duplicate determinations. Because the triacylglycerol content differed between experiments in the same cell type, the data in Expt. 1 and 2 were adjusted to the 8 or 10 hr time points, respectively, to compare the rates of triacylglycerol accumulation (factor for J774 = 0.4, smooth muscle cells = 2.5).

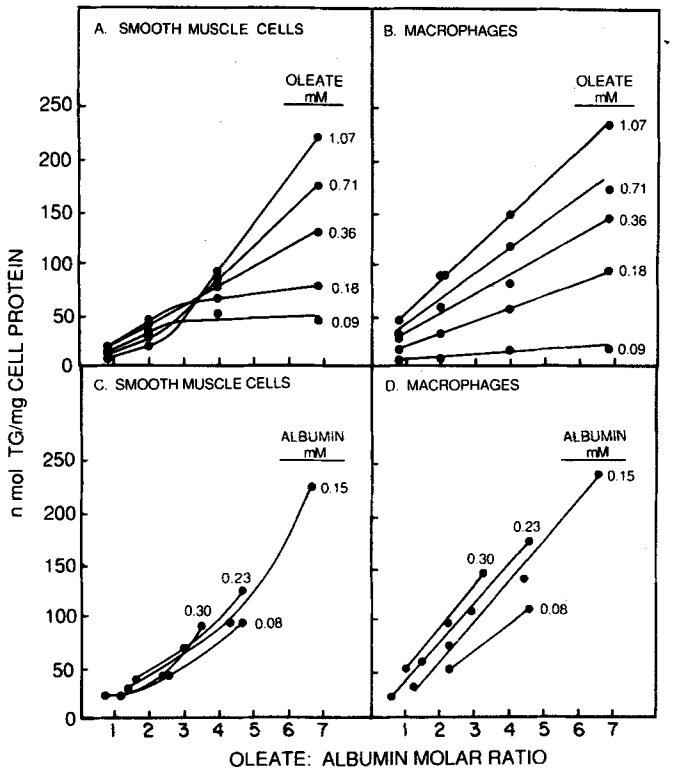

FIG. 4. Effect of oleate and albumin concentration at various oleate to albumin molar ratios on intracellular triacylglycerol content. Smooth muscle cells (A and C) and J774 macrophages (B and D) were incubated for 24 hr with the indicated concentration of oleate complexed to albumin at several molar ratios. Average of six experiments, each performed in duplicate. FA, fatty acid (oleate); BSA, bovine serum albumin; TG, triacylglycerol. The data from the same experiments are plotted in terms of the oleate (A and B) or albumin (C and D) concentration in the media.

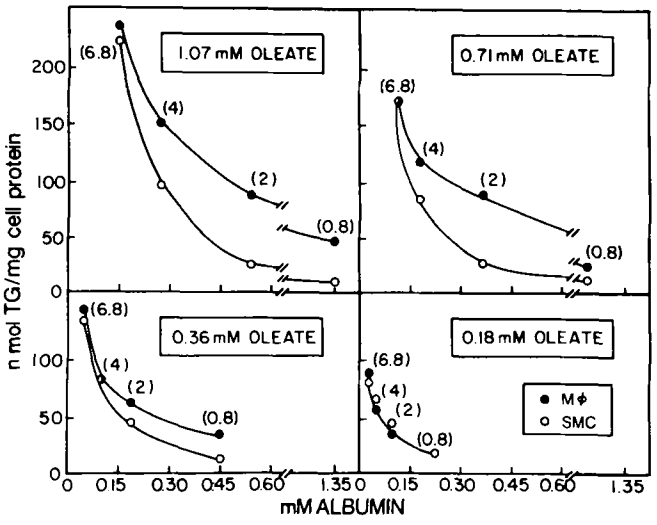

FIG. 5. The effect of increasing media albumin concentrations at a constant oleate concentration on the extent of triacylglycerol accumulation in macrophages and smooth muscle cells. The data are replotted from Figure 4. The molar ratio of oleate to albumin is in parentheses.

The relationship of the exogenous oleic acid concentration to the extent of intracellular triacylglycerol accumulation was complex. At a ratio of 6.8 mol of oleic acid per mole of albumin, smooth muscle cell triacylglycerol content (Fig. 4A) was linearly related to the exogenous oleate concentration up to 0.36 mM oleate. Greater amounts of oleate in the medium continued to promote higher levels of intracellular triacylglycerol, although to a lesser extent, with no indication of saturation. A similar result was shown in macrophages (Fig. 4B, oleate/albumin, 6.8:1).

At oleate to albumin ratios less than 6.8 to 1, the pattern of triacylglycerol accumulation showed three important differences between the two cell types. First, at the two lowest fatty acid to albumin molar ratios, 0.8:1 and 2:1, the smooth muscle cells contained more triacylglycerol at 0.18 mM oleate than at a six-fold higher concentration of oleate, 1.07 mM oleate (Fig. 4A). With macrophages (Fig. 4B), on the other hand, as the medium concentration of oleate increased, the triacylglycerol content of the macrophages rose accordingly, regardless of the oleate to albumin molar ratio. Second, it has been shown that more fatty acid is loosely bound to albumin at a 6.8:1 molar ratio of fatty acid to albumin than at a 2:1 molar ratio (39). By replotting the data holding the concentration of albumin constant, it became clear that the accumulation of smooth muscle cell triacylglycerol (Fig. C) was parallel to the expected amount of fatty acid loosely bound to albumin and thus available for uptake by the cells. This was not the case for macrophages (Fig. D). Finally, Figure 5 directly compares the triacylglycerol enrichment of smooth muscle cells and macrophages at equivalent levels of oleate and albumin. It was apparent that the response of the two cell types to the presence of exogenous albumin also differed. At constant oleate concentrations in the media of 1.07, 0.71 or 0.36 mM (Fig. 5), as the level of albumin is raised, the amount of triacylglycerol in both cells decreased. However, at concentrations of oleate of 0.36 mM or greater and at levels of albumin higher than 0.15 mM, macrophages contained 2.6-fold more triacylglycerol than smooth muscle cells (range = 1.4–5.0).

From the results in Figure 5, it appeared that one of the factors making a contribution to the regulation of intracellular triacylglycerol levels in the two cell types was a difference in their response to albumin in the medium. To examine this question directly, the two cell types initially were exposed to oleate to load the cells with triacylglycerol. When subsequently exposed to 0.15 mM albumin for 24 hr, smooth muscle cells preloaded with triacylglycerol demonstrated a greater loss of lipid (80%) than did similarly treated macrophages (10%), as shown in Table 1.

Differences in the triacylglycerol metabolism between the two cell types also could be attributed to a variation in their uptake of fatty acid. Thus, smooth muscle cells and macrophages were incubated with labeled oleate at a high oleate to albumin molar ratio (6.8 to 1) to reduce the effect of albumin. Early time points were chosen to minimize alterations in specific activity due to the hydrolysis of lipids. The results in Table 2 indicate that the labeled fatty acid was rapidly incorporated into phospholipid and triacylglycerol by both cells. However, in the first two hr, twice as much oleate was taken up by the J774 macrophages as compared with the smooth muscle cells, with the differences between the two cells reflected in the greater incorporation of oleate into triacylglycerol and phospholipid in macrophages.

DISCUSSION

This study has demonstrated that the metabolism of fatty acids by macrophages and smooth muscle cells have some common characteristics although there are significant quantitative differences. Exposure of both cell types to oleic acid at 0.18 mM (50 μg/ml) or greater and at a 2:1 oleate to albumin molar ratio or greater produced substantial intracellular triacylglycerol accumulation. Such a response to elevated media fatty acid concentrations has been found in a number of studies using other cell types (37,38). The pattern of triacylglycerol accumulation over time for macrophages and smooth muscle cells also was similar. Both cell types became enriched in

TABLE 1

Loss of Cellular Triacylglycerol Due to Incubation with Albumin

	n mol TG/mg cell protein			
		24 hr with:		% Loss of TG due to albumin
Cell	0 hr	DMEM (a)	Albumin (b)	(b)−(a) / (a)
J774	121 ± 5	91 ± 8	82 ± 12	10%
SMC	98 ± 7	115 ± 10	23 ± 19	80%

J774 and smooth muscle cells were loaded with triacylglycerol by incubation with 0.71 mM oleate (oleate:BSA molar ratio of 4:1, 0.18 mM BSA) for 24 hr = 0 hr. These media were removed, and the cells were incubated for an additional 24 hr in 0.15 mM BSA. The results represent the mean ± SD of two experiments performed in duplicate or triplicate. TG, triacylglycerol.

TABLE 2

Uptake of Labeled Free Fatty Acid by Smooth Muscle Cells and Macrophages

Hour	Cell	cpm × 10^{-3} in lipid/mg cell protein				
		TG	PL	FFA	CE	Total
1	SMC	18.0 ± 1.6	6.6 ± 0.6	3.2 ± 0.3	0.1 ± 0.0	27.9 ± 2.5
	J774	39.3 ± 4.4	12.5 ± 0.6	3.4 ± 0.6	1.8 ± 0.2	57.0 ± 5.4
2	SMC	26.7 ± 5.6	11.2 ± 0.8	2.0 ± 0.3	0.1 ± 0.0	40.0 ± 6.6
	J774	56.0 ± 7.1	18.1 ± 2.5	3.1 ± 0.2	3.2 ± 0.2	80.4 ± 8.8

Smooth muscle cells (SMC) and macrophages (J774) were incubated for the indicated time period with 0.71 mM ^{14}C-oleate (oleate:albumin molar ratio of 6.8:1). TG, triacylglycerol; PL, phospholipid; FFA, free fatty acid; CE, cholesteryl ester; total, sum of the ^{14}C-oleate cpm incorporated into the lipids. The data are the mean ± SD of triplicate determinations and are representative of the four experiments performed.

triacylglycerol in three phases, an initial slow rise, followed by a rapid increase and a plateau.

Other aspects of fatty acid metabolism by the two cell types were quite different. The extent of triacylglycerol enrichment was more pronounced in the J774 macrophages than in monkey smooth muscle cells under conditions in which the albumin concentration in the medium was greater than 0.15 mM and the oleate concentration was greater than 0.18 mM (Fig. 5). Differences in the degree of triacylglycerol accumulation between different cell types also was noted in a comparative study of human fibroblasts and guinea pig smooth muscle cells where fibroblasts were less reactive than the smooth muscle cells (38).

The extent of triacylglycerol accumulation in cells exposed to free fatty acid bound to albumin is controlled by several factors that govern the uptake of free fatty acid, the activity of the triacylglycerol synthetase and the mobilization of intracellular triacylglycerol. The dissociation of fatty acid from albumin currently is felt to be the rate-limiting step for the uptake of fatty acids by cells (40). Serum albumin binds fatty acid via six binding sites involving three primary and three secondary sites (39). At a constant free fatty acid concentration, a greater proportion of free fatty acid would be associated with albumin via lower affinity sites at a 6.8 to 1 molar ratio of fatty acid to albumin than would be the case at a 2:1 molar ratio. The uptake of fatty acids by cells was shown to be related to the concentration of loosely bound fatty acid in a study examining the one-min uptake of a fatty acid analogue, 3,3,12,12-tetramethylmyristic acid (41). The uptake of the analogue was shown to have a curvilinear relationship to the medium fatty acid to albumin molar ratio at either a constant albumin or a constant fatty acid medium concentration and was greater at the higher fatty acid to albumin molar ratios (41). The accumulation of triacylglycerol in smooth muscle cells at fixed albumin concentrations up to 0.3 mM showed the same curvilinear relationship with increasing oleate: albumin molar ratios (Fig. 4C). Such data might indicate that the accumulation of triacylglycerol in the smooth muscle cells was related to the availability of loosely bound free fatty acid in the media. However, only at the higher fixed oleate concentrations (0.71 and 1.07 mM) was the oleate/albumin molar ratio related in curvilinear fashion to the triglyceride accumulation (Fig. 4A). At the lower oleate concentrations, the exogenous levels of oleate were not sufficient to promote further triacylglycerol accumulation even when the amount of unbound fatty acid was elevated by increasing the fatty acid to albumin molar ratio.

Macrophages responded quite differently to these challenges than smooth muscle cells. Macrophage triacylglycerol content was linearly related to the oleate to albumin molar ratio at both a constant albumin concentration (Fig. 4D) and a constant oleate concentration (Fig. 4B). These data suggest that the accumulation of triacylglycerol in macrophages was related to the total amount of free fatty acid in the media, regardless of its binding affinity to albumin. One possible explanation for these observations is that unlike the smooth muscle cell, the macrophage takes up the albumin and fatty acid complex intact and that the fatty acid only leaves the complex once the latter is inside the cell.

The hydrolysis of cellular triacylglycerol and its mobilization from the cell is felt to be regulated by the concentration of exogenous albumin, which functions as a carrier for the released free fatty acid. At a constant oleate concentration, both cells contained less triacylglycerol as the levels of albumin in the media increased (Fig. 5), but this effect was more pronounced in smooth muscle cells. The ability of albumin to mobilize more triacylglycerol from smooth muscle cells than from macrophages may have contributed to the differences in intracellular triacylglycerol levels observed in these two cell types. The mechanism for the mobilization of triacylglycerol from cytoplasmic droplets is not clear. Most cells contain an acid acylhydrolase in lysosomes. In addition, macrophages have been shown to contain an active neutral triglyceride lipase in the cytosol (42). Loss of intracellular triacylglycerol with a concomitant appearance of free fatty acid in the media in the presence of exogenous albumin has been demonstrated in both J774 and mouse peritoneal macrophages (19,30). Whether smooth muscle cells also contain a neutral lipase and the relative sensitivity of both enzymes to the presence of exogenous albumin in the two cell types remains to be determined.

In summary, in comparison with smooth muscle cells, macrophages were shown to incorporate more free fatty acid over a short time period, accumulate more triacylglycerol under certain conditions and, upon exposure to

albumin, retain more triacylglycerol. In addition, macrophage triacylglycerol accumulation was linearly related to the oleate:albumin molar ratio, whereas smooth muscle cell triacylglycerol accumulation showed a curvilinear relationship. The mechanism for such differences between the two cell types remains to be elucidated. Although differences in the activity of intracellular lipases are a possibility, another interesting alternative involves the fatty acid-binding "Z" protein (5,6). The results are consistent with the hypothesis that the macrophage "Z" protein has a higher affinity for fatty acids than does the smooth muscle cell protein or that there is more "Z" protein in macrophages. Over a two-hr time period at a high oleate concentration to rapidly saturate the "Z" protein, macrophages incorporated twice as much labeled fatty acid as smooth muscle cells. Smooth muscle cells, which readily lost their intracellular triglyceride as fatty acid to albumin, did not appear to take up the fatty acid bound with high affinity to albumin. Finally, macrophages, which did not readily give up intracellular fatty acid to albumin from triacylglycerol hydrolysis, also were able to take up fatty acid from albumin in the media regardless of the molar ratio of fatty acid to albumin.

It was of interest that saturated fatty acids (palmitate, 16:0) supported a much lower level of triacylglycerol accumulation in smooth muscle cells than did unsaturated fatty acids (oleate, 18:1 and linoleate, 18:2). Palmitate binds more tightly to albumin than oleate or linoleate (39), making it less available to the cell for triacylglycerol synthesis. Other studies also have shown that the degree of saturation of fatty acids will influence the degree of triacylglycerol synthesis and accumulation (37,38,43).

Evidence that changes in fatty acids occur with atherosclerosis is accumulating (7-17). Whether the presence of free fatty acids in the arterial wall or their ability to promote the accumulation of intracellular triglycerides contributes to the atherogenic process remains to be determined. We have found that the presence of oleate is associated with a further increase in the cholesteryl ester content of smooth muscle cells exposed to hyperlipemic LDL (data not shown). Recently, an increased triacylglycerol content has been shown to influence the rate of clearance of cholesteryl esters from rat hepatomas (20) and the rate of transport of albumin across endothelial cells (44).

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REFERENCES

1. Howard, B.V., and Kritchevsky, D. (1969) *Biochim. Biophys. Acta 187,* 293-301.
2. Dole, V.P. (1956) *Clin. Invest. 35,* 150-154.
3. Gordon, R.S. Jr., and Cherkes, A. (1956) *J. Clin. Invest. 35,* 206-212.
4. Spector, A.A. (1968) *Ann. N.Y. Acad. Sci. 149,* 768-783.
5. Mishkin, S., Stein, L., Gatmaitan, Z., and Arias, I.M. (1972) *Biochem. Biophys. Res. Commun. 47,* 997-1002.
6. Ockner, R.K., Manning, J.A., Popenhausen, R.B., and Ho, W.K.L. (1972) *Science 177,* 56-58.
7. Pickart, L. (1983) *Atherosclerosis 46,* 21-28.
8. Schrade, W., Biegler, R., and Bohle, E. (1961) *J. Atheroscl. Res. 1,* 47-53.
9. Inoue, S., Ohta, M., Iizuka, T., and Murao, S. (1975) *Jpn. Heart J. 16,* 670-678.
10. Gordon, J.L., Bowyer, D.E., Evans, D.W., and Mitchinson, M.J. (1973) *J. Clin. Pathol. 26,* 958-962.
11. Kershbaum, A., Bellet, S., Dickstein, E.R., and Fienberg, L.J. (1961) *Circ. Res. 9,* 631-638.
12. Bell, F.P., Adamson, I.L., and Schwartz, C.J. (1974) *Exp. Mol. Pathol. 20,* 57-68.
13. Adams, C.W.M., Morgan, R.S., and Bayliss, O.B. (1970) *Atherosclerosis 11,* 119-124.
14. Corey, J.E., and Zilversmit, D.B. (1977) *Atherosclerosis 27,* 201-212.
15. Schaffner, T., Taylor, K., Bartucci, E.J., Fischer-Dzoga, K., Beeson, J.H., Glagov, S., and Wissler, R.W. (1980) *Am. J. Pathol. 100,* 57-80.
16. Fowler, S., Shio, H., and Haley, N.J. (1979) *Lab. Invest. 41,* 372-378.
17. Khoo, J.C., Mahoney, E.M., and Witztum, J.L. (1981) *J. Biol. Chem. 256,* 7105-7107.
18. Bates, S.R., Murphy, P.L., Feng, Z., Kanazawa, T., and Getz, G.S. (1984) *Arteriosclerosis 4,* 103-114.
19. Bergstraesser, L.M., and Bates, S.R. (1985) *Biochim. Biophys. Acta 836,* 296-305.
20. Adelman, S.J., Glick, J.M., Phillips, M.C., and Rothblat, G.H. (1984) *J. Biol. Chem. 259,* 13844-13850.
21. Goldstein, J.L., and Brown, M.S. (1975) *Arch. Path. 99,* 181-184.
22. Eisele, B., Bates, S.R., and Wissler, R.W. (1980) *Atherosclerosis 36,* 9-24.
23. St. Clair, R.W., Mitschelen, J.J., and Leight, M.A. (1980) *Biochim. Biophys. Acta 618,* 63-79.
24. Goldstein, J.L., Ho, Y.K., Brown, M.S., Innerarity, T.L., and Mahley, R.W. (1980) *J. Lipid Res. 21,* 970-980.
25. Fischer-Dzoga, K., Jones, R.M., Vesselinovitch, D., and Wissler, R.W. (1973) *Exp. Mol. Pathol. 18,* 162-176.
26. Bates, S.R. (1980) *Artery 7,* 303-315.
27. Ralph, P., Prichard, J., and Cohn, M. (1975) *J. Immunol. 114,* 898-905.
28. Khoo, J.C., Mahoney, E.M., and Steinberg, D. (1981) *J. Biol. Chem. 256,* 7105-7107.
29. Bates, S.R., Coughlin, B.A., Mazzone, T., Borensztajn, J., and Getz, G.S. (1987) *J. Lipid Res. 28,* 787-797.
30. von Hodenberg, E., Khoo, J.C., Jensen, D., Witztum, J.L., and Steinberg, D. (1984) *Arteriosclerosis 4,* 630-635.
31. Via, D.P., Plant, A.L., Craig, I.F., Gotto, A.M., and Smith, L.C. (1985) *Biochim. Biophys. Acta 833,* 417-428.
32. St. Clair, R.W., Smith, B.P., and Wood, L.L. (1977) *Circ. Res. 40,* 166-173.
33. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
34. Marsh, J.B., and Weinstein, D.B. (1966) *J. Lipid Res. 7,* 574-576.
35. Kritchevsky, D., Davidson, L.M., Kim, H.K., and Malhotra, S. (1973) *Clin. Acta 46,* 63-68.
36. Lowry, O.H., Rosebrough, N.J., Farr, L.A., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
37. Denning, G.M., Figard, P.H., Kaduce, T.L., and Spector, A.A. (1983) *J. Lipid Res. 24,* 993-1001.
38. Gavino, V.C., Miller, J.S., Dillman, J.M., Milo, G.E., and Cornwell, D.G. (1981) *J. Lipid Res. 22,* 57-62.
39. Spector, A.A., John, K., and Fletcher, J.E. (1969) *J. Lipid Res. 10,* 56-66.
40. Weisiger, R.A., and Ma, W-L. (1987) *J. Clin. Invest. 79,* 1070-1077.
41. Spector, A.A., Steinberg, D., and Tanaka, A. (1965) *J. Biol. Chem. 240,* 1032-1041.
42. Khoo, J.C., Vance, J.E., Mahoney, E.M., Jensen, D., Wancewicz, E., and Steinberg, D. (1984) *Arteriosclerosis 4,* 34-40.
43. Rosenthal, M.D. (1981) *Lipids 16,* 173-182.
44. Henning, B., Shasby, D.M., Fulton, A.B., and Spector, A.A. (1980) *Arteriosclerosis 4,* 489-497.

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Influence of Dietary Soybean and Egg Lecithins on Lipid Responses in Cholesterol-fed Guinea Pigs[1]

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The comparative influence on plasma and tissue lipids of dietary soybean and egg lecithins, which have contrasting fatty acid compositions, was studied in the hypercholesterolemic guinea pig. The polyunsaturated to saturated fatty acid (P/S) ratios of the soybean and egg lecithins were 3.4 and 0.38, respectively. Hypercholesterolemia was induced by feeding guinea pigs a purified diet that contained 15% lard enriched with 0.5% cholesterol. Subsequently, guinea pigs were fed for six wk the same diet supplemented with either soybean or egg lecithin as 7.5% of the diet. A control group continued to be fed the lecithin-free diet. Parameters measured included body weight and relative liver weight; in plasma, total cholesterol, high density lipoprotein cholesterol (HDLC), phospholipid, and nonesterified cholesterol; in liver, total fat, cholesterol, and the specific activity of the catabolic enzyme cholesterol 7α-hydroxylase; (EC 1.14.13.17); and in the aorta, cholesterol. Among the most noteworthy observations were the 49% decrease in total plasma cholesterol of the soybean lecithin group without decreasing HDLC and the 177% increase in HDLC of the egg lecithin group without a significant increase in total cholesterol compared with those values in the control group. These data suggest that dietary lecithin is particularly effective in increasing the HDLC/total cholesterol ratio in plasma. However, the absolute concentrations of those plasma lipids seem to depend upon the fatty acid composition of the lecithin.
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Lecithin, primarily from soybeans, is widely used as a hypocholesterolemic diet supplement, although experimental evidence to support that use is inconsistent (1–3). More recent observations suggest that soybean lecithin has greater effectiveness in increasing high density lipoprotein (HDL) cholesterol than in lowering total plasma cholesterol (4–6). This is somewhat surprising because soybean lecithin contains a high level (60–70%) of polyunsaturated fatty acids (PUFA), and dietary PUFA incorporated into triglycerides tend to lower HDL cholesterol (7). However, Childs et al. (5) have presented data that suggest dietary soybean lecithin elicits some plasma lipid responses that are independent of its fatty acid composition.

Eggs are a rich source of dietary lecithin, which is approximately 6.8% (w/w) of a chicken egg yolk (8). The fatty acid composition of egg lecithin is in sharp contrast to that of soybean lecithin as revealed by the polyunsaturated to saturated fatty acid (P/S) ratios of these two sources of dietary lecithin: soybean, 3.4; egg, 0.38.

This investigation was designed to compare the influence of soybean and egg lecithins on plasma lipids and lipoproteins and on the cholesterol contents of liver and aorta in hypercholesterolemic guinea pigs. The guinea pig was chosen as experimental animal because of its normally low HDL, which is similar in composition to human HDL (9).

MATERIALS AND METHODS

Animals. Female English shorthair guinea pigs (Hilltop Laboratory Animals, Scottdale, PA) weighing 400–500 g were housed in polycarbonate cages in an environmentally controlled room that was illuminated from 0600 to 1800 hr.

Frequently, guinea pigs will starve rather than eat purified diets. The mature guinea pigs used in this study were trained to accept a purified diet (20–25 g/day) by gradually changing their food from stock guinea pig diet (Harlan/Sprague Dawley Co., Inc., Madison, WI) to purified diet. During the two-wk period before the experiment began, the guinea pigs were fed, sequentially, ground mixtures of stock/purified (w/w) that were 3:1, 1:1, 1:3, and finally the unadulterated purified diet. All the guinea pigs followed the same training time schedule. They maintained their body weights during the training period.

Diets. The diets were prepared commercially (Dyets, Inc., Bethlehem, PA) according to the formulations shown in Table 1. The cholesterol-free diet (BAS) was used in training the guinea pigs to accept a purified diet. The cholesterol-supplemented diet of purified ingredients without lecithin (NL) was used to induce hypercholesterolemia in the guinea pigs. Other investigators, who used 1% or 1.6% dietary cholesterol, observed aberrant serum lipoprotein responses in the guinea pig (10–12). The level of dietary cholesterol in this study, 0.5%, was judged to be sufficient to produce hypercholesterolemia and to be consistent with the level used by Wong et al. (4) in studying the effect of dietary soybean lecithin on plasma lipids of hyperlipemic rhesus monkeys.

For the two lecithin-containing diets, lecithin replaced 4% of the lard and 3.5% of the sucrose compared to the NL diet. The dietary lecithins were from soybeans (SL diet) and from eggs (EL diet) and were provided by the American Lecithin Company (Atlanta, GA) and the Asahi Chemical Industry Company (Tokyo), respectively. The phospholipid and fatty acid compositions of the soybean and egg lecithins are given in Table 2.

Because phospholipids are highly susceptible to oxidation (15), the diets were stored at −20 C. The diets were assayed weekly for malondialdehyde (MDA), a peroxidation product, using thiobarbituric acid. In preliminary testing, the lecithin-containing diets were assayed for MDA content following exposure to ambient conditions for 24 and 48 hr. MDA was present in measurable quantities only after 48 hr.

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Abbreviations: BAS, cholesterol-free diet; EDTA, ethylenediamine tetraacetic acid; EL, egg lecithins; FC, nonesterified cholesterol; HDL, high density lipoproteins; HDLC, high density lipoprotein cholesterol; MDA, malondialdehyde; NL, lecithin-free; PL, phospholipids; PUFA, polyunsaturated fatty acids; SL, soybean lecithins; TC, total plasma cholesterol; VLDL, very low density lipoprotein.

TABLE 1

Compositions of Purified Diets[a]

Ingredient	g/kg diet			
	BAS	NL	SL	EL
Soy protein isolate	200	200	200	200
Cornstarch	100	100	100	100
Sucrose	306	301	266	266
Cellulose	150	150	150	150
Lard	150	150	110	110
Mineral mix[b]	75	75	75	75
Vitamin mix[c]	10	10	10	10
DL-Methionine	3	3	3	3
Choline bitartrate	2	2	2	2
Ascorbic acid	4	4	4	4
Cholesterol	—	5	5	5
Soybean lecithin[d]	—	—	75	—
Egg lecithin[e]	—	—	—	75

[a]Prepared by Dyets, Inc. (Bethlehem, PA).

[b]Mineral mix (g/kg mix): calcium carbonate, 65.56; calcium dihydrogen phosphate, 310.0; sodium chloride, 118.0; potassium citrate, 325.0; potassium sulfate, 89.5; magnesium oxide, 59.0; manganous carbonate, 2.47; ferric citrate (U.S.P.), 4.7; zinc carbonate, 0.71; cobalt carbonate, 0.03; cupric carbonate, 0.12; potassium iodide, 0.03; sodium selenite, 0.003; chromium potassium sulfate, 0.07; sucrose, 24.807.

[c]Vitamin mix (g/kg mix): thiamine HCL, 3.2; calcium pantothenate, 8.0; riboflavin, 3.2; pyridoxine HCL, 3.2; niacin, 40.0; biotin, 0.12; folic acid, 2.0; vitamin B12 (0.1%), 8.0; vitamin A palmitate (500,000 IU/g), 6.91; vitamin D2 (500,000 IU/g), 0.64; vitamin E acetate (500 IU/g), 8.0; menadione (sodium bisulfite), 0.64; inositol, 400.0; sucrose, 516.09.

[d]Courtesy of the American Lecithin Company (Atlanta, GA).

[e]Courtesy of Asahi Chemical Industry Co., Ltd. (Tokyo).

TABLE 2

Phospholipid and Fatty Acid Compositions of Lecithins

Lipid components	Lecithin source	
	Soybean (%)[a]	Egg (%)[b]
Phospholipid[a]		
Phosphatidylcholine	51	59
Phosphatidylethanolamine	25	19
Lysophosphatidylcholine	9	2
Fatty acid[b]		
16:0	17.3	31.9
16:1 (n-7)	—	2.5
18:0	3.6	15.7
18:1 (n-9)	10.1	31.6
18:2 (n-6)	62.5	14.6
18:3 (n-3)	6.5	0.6
20:4 (n-6)	—	3.0

[a]Analyses according to Rouser et al. (13).

[b]Determined by gas liquid chromatography (14).

Experimental design and methods. Guinea pigs that had been trained to accept a purified diet were fed the NL diet for six wk to induce hypercholesterolemia. The animals then were randomly assigned to one of three diet groups (eight guinea pigs/group): SL, EL or NL (control group) and fed the assigned diet for six wk. Fresh food was provided daily. Body weights were recorded weekly.

The guinea pigs were anesthetized with carbon dioxide and killed by decapitation. Blood was collected from the neck wound into chilled centrifuge tubes containing 1 mg/ml ethylenediamine tetraacetic acid disodium salt (EDTA) as anticoagulant. Livers were rapidly excised and placed in ice-cold beakers. Aortas were removed intact from the heart to approximately 1 cm below the point of bifurcation and placed in 0.15 M NaCl. Plasma was isolated by centrifugation (2,000 × g) at 5 C for 30 minutes. The HDL fraction was separated from 1-ml aliquots of plasma following manganese-heparin precipitation of very low density (VLDL) and low density lipoproteins (LDL) (16). Total lipids were extracted from 1 ml of plasma according to Kates for the determination of nonesterified cholesterol (FC) and phospholipids (PL) (17). Plasma FC was separated as the digitonide (18). Total plasma cholesterol (TC), FC and HDL cholesterol (HDLC) were determined spectrophotometrically as described (19). Plasma PL were determined spectrophotometrically according to Rouser et al. (13).

For the isolation of microsomes, a six-gram portion of liver was homogenized with 5 volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 20 mM nicotinamide and 1.0 mM EDTA with a Potter-Elvehjem homogenizer equipped with a teflon pestle. Microsomes were prepared from this liver homogenate as described by Mitropoulos and Balasubramaniam (20); the microsomal enzyme cholesterol 7α-hydroxylase (EC 1.14.13.17) was assayed according to Hassan et al. (21). The remainder of the liver tissue was frozen at −20 C to be used later for the determination of total fat and cholesterol.

The liver fat was extracted by the method of Folch (22) and determined gravimetrically. Cholesterol was determined in the lipid extract (1%, w/v, chloroform solution) by the method cited above for plasma.

Aortas were carefully stripped of adventitial fat and other adherent tissue. Aortas from half of the animals in each treatment group were preserved and stained with a lipophilic stain (23). Cholesterol also was determined after the weighed aortas were digested in refluxing 12% (w/w) ethanolic KOH for one hr. Cholesterol was determined in the hexane extract of the nonsaponifiable fraction (16).

Student's t-test was used to evaluate the significance of differences between the SL and NL treatments and between the EL and NL treatments (24).

RESULTS AND DISCUSSION

The final body weights and relative liver weights (as % body weight) for the NL, SL and EL treatment groups are given in Table 3. Although whole body weight did not appear to depend on diet composition, liver weights were significantly higher in animals consuming the EL diet.

Average plasma lipid concentrations are presented in Table 3. Guinea pigs that were fed only the NL diet for the entire treatment period (12 wk) had an average TC of 265 ± 39 mg/dL, a three-fold increase assuming an initial TC of 81 mg/dL, the value reported for stock-fed guinea pigs (10). The SL diet, but not the EL diet, attenuated that increase: SL, 131 ± 19 mg/dL and EL,

TABLE 3

Hypercholesterolemic Guinea Pigs Fed Dietary Cholesterol without and with Either Soybean or Egg Lecithin

Parameter	Dietary tratment[a]		
	NL[b]	SL[b]	EL[b]
Body weight (g)	670 ± 19[c]	676 ± 18	687 ± 14
Liver weight (% Body wt)	5.9 ± 0.4	8.7 ± 1.3	12.7 ± 0.5*
Plasma			
TC (mg/dL)	265 ± 39	131 ± 19*	291 ± 35
FC (mg/dL)	136 ± 6	63 ± 8*	148 ± 8
HDLC (mg/dL)	47 ± 6	58 ± 13	130 ± 20*
PL (mg/dL)	46 ± 11	28 ± 10	70 ± 18
Liver			
Total fat (mg/g)	130 ± 22	142 ± 31	184 ± 14**
TC (mg/g)	20.0 ± 2.5	25.6 ± 2.8	29.6 ± 1.7*
Chol 7α-OH $\left(\frac{\text{pmol produced}}{\text{mg protein} \cdot 20\ \text{min}}\right)$	0.63 ± 0.17	0.52 ± 0.17	0.72 ± 0.10
Aorta			
TC (mg/g)	4.9 ± 0.9	2.9 ± 0.8**	5.6 ± 0.8

TC, total cholesterol; FC, nonesterified cholesterol; HDLC, high density lipoprotein cholesterol; PL, phospholipids; Chol 7α-OH, cholesterol 7α-hydroxylase.

[a]See Materials and Methods for details.

[b]NL, lecithin-free diet; SL, diet containing 7.5% soybean lecithin; EL, diet containing 7.5% egg lecithin.

[c]Mean ± SEM, values represent average of four to eight animals.

*$P < 0.05$ compared with NL.

**$0.05 < P < 0.10$ compared with NL.

291 ± 35 mg/dL. The EL diet had a dramatic effect on HDLC. Guinea pigs fed only on the NL diet had an average HDLC level of 47 ± 6 mg/dL. Hypercholesterolemic guinea pigs fed the SL diet for six wk had HDLC of 58 ± 13 mg/dL, which is a change of marginal significance. However the EL diet elicited a 2.8-fold increase in HDLC to 130 ± 20 mg/dL. It is interesting to note that the ratio of HDLC/TC in plasma is 0.44 and 0.45 for the SL and EL groups, respectively, whereas that ratio is 0.18 for the NL group.

The TC response in guinea pigs fed the SL diet and the HDLC response in guinea pigs fed the EL diet were qualitatively consistent with the expected responses to polyunsaturated and saturated diet fats, respectively. That is, polyunsaturated diet fat tends to lower TC, and saturated diet fat tends to increase HDLC (25). However, the magnitude of the effect of the SL diet on TC exceeded what one would expect from the relatively small contribution of polyunsaturated fat (5.2%) to the diet from soybean lecithin. Likewise, the 3.6% saturated fatty acid esters in the diet from in the egg lecithin probably would not result in the enormous increase in HDLC if that quantity were added as dietary triglycerides. Other investigators have supported that prediction. Childs et al. (5) could not account for the effects of ingested soybean lecithin on TC and HDLC in normolipidemic humans by replacing the dietary lecithin with triglycerides of equivalent fatty acid composition. Other workers have reported nonequivalent hypocholesterolemic responses when rats ingested soybean lecithin compared with the effect of either soybean triglycerides (26) or corn oil (27).

Dietary lecithin (phosphatidylcholine) is not metabolized like dietary triglycerides. In other words, a mole of lecithin is not equivalent to two-thirds of a mole of triglycerides. Dietary lecithin is hydrolyzed to lysolecithin and fatty acids by phospholipase A_2 in the intestines before absorption (28). Diagne et al. (29) have shown that much of the absorbed lysolecithin is reacylated to the corresponding lecithin in guinea pigs. When Beil and Grundy (27) studied the effects of ingested lecithin on lipoproteins in humans, they found that lecithin, in contrast with dietary triglycerides, promotes VLDL formation, and VLDL is a source of phospholipids and apoprotein for HDL (30). Thus, one might predict an increase in HDLC in response to dietary lecithin, but one would not predict the high level of HDLC in the EL group. As with the hypocholesterolemic response when guinea pigs were fed the SL diet, fatty acids incorporated into lecithins seemed to have an exaggerated effect compared with their effect when ingested as triglycerides.

Included in Table 3 are average plasma concentrations of FC and PL. Because the plasma lipid level was very low for SL-fed animals, the lipid extract from the plasma that was available was insufficient to determine both FC and PL. Therefore, the values of FC and PL shown for the SL group were each the average of four different

guinea pigs. The proportion of esterified plasma cholesterol was not altered by these diet modifications. These data suggest an increase in the PL/FC ratio. Of course, that conclusion is particularly questionable because the components of the ratio were determined from different animals in the diet group. When Wong et al. (4) fed soybean lecithin (92% phosphatidylcholine) to hyperlipemic rhesus monkeys, they observed a reduction in total plasma lipids, especially in LDL cholesterol, but a marginal increase in HDLC and a possible increase in the PL/FC ratio. The results of this investigation using hyperlipemic guinea pigs fed soybean lecithin were qualitatively consistent with those results. In both investigations, highly variable individual responses to lecithin were observed.

The specific activities of cholesterol 7α-hydroxylase (hydroxylase) for groups NL, SL and EL also are included in Table 3. Neither treatment group SL nor EL were significantly different from the control, NL. The inhibition of cholesterol absorption by dietary lecithin has been reported in humans (31) and in rats (32). Although the effect of dietary lecithin on cholesterol absorption could be reflected in a coordinate change in hydroxylase activity (19), these hydroxylase activities (Table 3) suggest no such correlation.

Total liver lipid and liver cholesterol levels in these diet groups are summarized in Table 3. Cholesterol ingested as part of a purified diet tends to exacerbate liver fat and cholesterol accumulations in guinea pigs (22) and in rats (11), compared with the effect of stock diets. Egg lecithin induced a further highly significant increase in liver size that could not be attributed to increased fat accumulation. The total fat content of the EL livers was only marginally higher than that of the NL group. Liver cholesterol, however, was significantly increased in the EL group as compared with the NL group.

When aortas from treatment groups NL, SL and EL were exposed to a lipophilic dye, Sudan IV, no fatty deposits were apparent. Compared with guinea pigs fed the NL diet, dietary SL tended to lower aortic cholesterol, while dietary EL had no effect (Table 3). Aortic cholesterol levels tended to reflect plasma cholesterol levels, which could be the result of passive diffusion. However, the accumulation of cholesterol in the liver appeared to be a concerted process in guinea pigs fed cholesterol-supplemented diets, a process that was exacerbated by dietary EL. Although dietary SL elicited a significant decrease in plasma TC, there was no decrease in liver cholesterol in SL-fed guinea pigs, compared with the NL group.

The results of this investigation provide considerable evidence that dietary phospholipids play a significant role in HDL metabolism; a role that is different from that of dietary triglycerides. At the same time, the "dietary phospholipid effect" is modulated by the fatty acid composition of the dietary phospholipid. Further investigations of the mechanism of these interactions are required.

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REFERENCES

1. Kesaniemi, Y.A., and Grundy, S.M. (1986) *Am. J. Clin. Nutr. 43*, 98–107.
2. Greten, H., Raetzer, H., Stichl, A., and Schettler, G. (1980) *Atherosclerosis 36*, 81–88.
3. Beynen, A.C., and Van Gels, L.G. (1983) *Experientia 39*, 492–493.
4. Wong, E.K., Nicolosi, R.J., Low, P.A., Herd, J.A., and Hayes, K.C. (1980) *Lipids 15*, 428–433.
5. Childs, M.T., Bowlin, J.A., Ogilvie, J.T., Hazzard, W.R., and Albers, J.J. (1981) *Atherosclerosis 38*, 217–228.
6. Hunt, C.E., and Duncan, L.A. (1985) *Br. J. Exp. Pathol. 66*, 35–46.
7. Mattson, F.H., and Grundy, S.M. (1983) *J. Lipid Res. 26*, 194–202.
8. Weihrauch, J.L., and Son, Y-S. (1983) *J. Am. Oil Chem. Soc. 60*, 1971–1978.
9. Chapman, M.J. (1980) *J. Lipid Res. 21*, 789–853.
10. Ostwald, R., and Shannon, A. (1964) *Biochem. J. 91*, 146–154.
11. Puppione, D.L., Sardet, C., Yamanaka, W., Ostwald, R., and Nichols, A.V. (1971) *Biochem. Biophys. Acta 231*, 295–301.
12. Chapman, M.J., and Mills, G.L. (1977) *Biochem. J. 167*, 9–21.
13. Rouser, G., Slakotos, A., and Fleischer, S. (1966) *Lipids 1*, 85–86.
14. O'Brien, B.C., Skutches, C.L., Henderson, G.R., and Reiser, R. (1977) *J. Nutr. 107*, 1444–1454.
15. Pikul, J., Leszczynski, D.E., and Kummerow, F.A. (1983) *J. Agric. Food Chem. 31*, 1338–1342.
16. Warnick, G.R. and Albers, J.J. (1978) *J. Lipid Res. 19*, 65–76.
17. Kates, M. (1972) *Techniques of Lipidology. Isolation, Analysis and Identification of Lipids*, p. 352, North-Holland Publishing Co., Amsterdam.
18. Kates, M. (1972) *Techniques of Lipidology. Isolation, Analysis and Identification of Lipids*, p. 361, North-Holland Publishing Co., Amsterdam.
19. O'Brien, B.C. and Reiser, R. (1982) *J. Nutr. 112*, 1490–1497.
20. Mitropoulos, K.A., and Balasubramaniam, S. (1972) *Biochem. J. 128*, 1–9.
21. Hassan, A.S., Yunker, R.L., Sprinkle, D.J., and Subbiah, M.T.R. (1983) *J. Nutr. 113*, 986–995.
22. Raden, N.S. (1969) in *Methods in Enzymology* (Lowenstein, J.M., ed.) Vol. XIV, pp. 245–254, Academic Press, New York.
23. Holman, R.L., McGill, H.C. Jr., Strong, J.P., and Geer, J.C. (1958) *Lab. Invest. 7*, 42–47.
24. Snedecor, G.W. (1956) *Statistical Methods*, 5th edn., pp. 45–46, Iowa State University Press, Ames, IA.
25. Vega, G.L., Groszek, E., Wolf, R., and Grundy, S.M. (1982) *J. Lipid Res. 23*, 811–822.
26. Smarzumi, K., Murata, M., and Sugano, M. (1982) *J. Nutr. Science Vitaminol. 28*, 281–294.
27. Beil, F.U., and Grundy, S.M. (1980) *J. Lipid Res. 21*, 525–536.
28. Arnesjo, B., Nelsson, A., Barrowman, J., and Borgstrom, B. (1968) *Scand. J. Gastroenterol. 4*, 653–665.
29. Diagne, A., Mitjavila, S., Fauvel, J., Chap, H., and Douste-Blazy, L. (1987) *Lipids 22*, 33–40.
30. Tall, A., and Small, D. (1978) *N. Eng. J. Med. 229*, 1232–1236.
31. O'Mullane, J.E., and Hawthorne, J.N. (1982) *Atherosclerosis 45*, 81–90.
32. Hollander, D., and Morgan, D. (1980) *Lipids 15*, 395–400.

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Malondialdehyde-containing Proteins and Their Relationship to Vitamin E[1]

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A high molecular weight (Sephadex G-15 void volume), water-soluble, fluorescent material that was found to increase significantly in the mouse liver in response to vitamin E deficiency was separated into six proteins by high performance liquid chromatography (HPLC) using a TSK G2000 SW column. One of these proteins increased significantly in concentration due to vitamin E deficiency and had a molecular weight of 20,000 daltons. This protein was found to contain malondialdehyde, an end product of lipid peroxidation, attached to it presumably in a Schiff-base type structure with amino groups. This appears to be the first report in the literature of direct evidence that malondialdehyde is attached to protein in vivo. *Lipids 23,* 651–655 (1988).

The accumulation of fluorescent pigments in tissues of nearly all types of animals under stressful conditions such as vitamin E deficiency and aging is widely accepted (1–9). These compounds have been termed "lipofuscin" or "age" pigments and are believed to be metabolic end products of lipid peroxidation in living systems that may be undergoing steady decomposition or turnover (1, 10–13). They have been postulated as Schiff-base products formed by the condensation of primary amino groups of proteins, nucleic acids or amino acids and malondialdehyde (MDA), a by-product of lipid peroxidation in vitro and in biological systems (14). Due to their lipid origin and staining properties, initial separation and purification of these fluorescent pigments was attempted only from the organic solvent-soluble extract (10,15). The amount of fluorescent material extracted, however, did not totally account for the lipofuscin pigment concentration reported in some tissues (3). Recently, four vitamin E-related fluorescent compounds were reported to occur in the water-soluble portion of mouse tissues (16). The purpose of this study was to elucidate the characteristics and possible identity of one of these compounds, a large molecular weight material, especially as it relates to vitamin E in the diet and to in vivo lipid peroxidation.

MATERIALS AND METHODS

Animals, diets and extraction. Female weanling mice (Strain C57BL/6J, The Jackson Laboratories, Bar Harbor, ME) were fed a vitamin E-deficient or a vitamin E-supplemented diet (30 mg RRR-α-tocopheryl acetate/kg diet) as described (16). After 14 months, the mice were killed by decapitation. The livers were removed, washed with cold saline-phosphate solution, blotted with cheesecloth and frozen at −70 C until they were homogenized and extracted with chloroform/methanol (2:1) and water (16). Water-soluble fluorescent compounds were separated chromatographically using Sephadex G-15 or G-25 (Pharmacia Fine Chemicals, Piscataway, NJ), and initial fluorescence measurements were performed using an Aminco-Bowman Ratio Spectrophotofluorometer (American Instrument Co., Silver Spring, MD) standardized as described (16).

HPLC equipment. Instrumentation for HPLC consisted of a Model 210 injection valve (Beckman Instruments, Inc., Berkeley, CA), a Beckman Model 110A solvent metering pump, a Perkin-Elmer Model 650-10S fluorescence spectrophotometer (Norwalk, CT) equipped with a horizontal, flat, 20 μl capacity, quartz flow cell, a Model SP 8400 variable wavelength ultraviolet/visible detector (Spectra Physics, Arlington, IL) equipped with a 10 μl quartz flow cell and a Model SP4100 computing integrator (Spectra Physics). The analytical columns used were a 0.75 × 30 cm Micropak TSK G2000 SW column (Varian Associates, Inc., Walnut Creek, CA) and a Spherogel TSK G1000 PW column (0.75 × 30 cm, Altex Scientific, Inc., Berkeley, CA). Samples were injected with a 100 or a 200 μl blunt needle Hamilton syringe (Rainin Instrument Co., Inc., Woburn, MA).

Molecular weight determination. The TSK G2000 SW column was calibrated for molecular weight determinations by injecting the following protein standards: catalase (250,000 m.w.), bovine serum albumin (65,000 m.w.), β-lactoglobulin (35,000 m.w.), α-chymotrypsinogen-A (23,200 m.w.), and lysozyme (14,100 m.w.). All standards were eluted at 1 ml/min in 0.066 M phosphate, 0.3 M sodium chloride, 0.02% sodium azide (pH 7.0), and detected at 275 nm/350 nm using the Perkin-Elmer fluorometer. The void volume material (ca. 6 ml) from Sephadex G-15 column chromatography of liver extracts was collected, and 100 μl aliquots were injected onto the TSK G2000 SW column under these same conditions. Using a least squares fit equation to the linear portion of the graph for the standard proteins (log m.w. − 0.17811 × elution volume + 6.13513), the molecular weights of each of the six compounds separating on the TSK column from the Sephadex G-15 void volume peak were determined. All these proteins had excitation/emission maxima at 275 nm/350 nm and were quantified by peak height measurement for both vitamin E–sufficient and deficient groups of mice and compared using Student's two-tailed t-test (17).

Hydrolysis for release of MDA. Portions of liver tissue (0.3–0.4 g) were extracted as described (16). The filtered lyophilized water extracts were dissolved in 150 μl water, and 100 μl aliquots were directly injected onto the TSK G2000 SW column. The same protein materials that were observed above were separated and collected (0.4–0.5 ml each). The collected protein peaks from the water-soluble liver extracts were hydrolyzed as follows. Volumes of 400–1000 μl were removed, and the pH was adjusted to greater than 12.5 with concentrated NaOH. The samples were enclosed and placed in the dark at room temperature

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Abbreviations: MDA, malondialdehyde; HPLC, high performance liquid chromatography.

overnight. The samples were neutralized with concentrated HCl, and 200 μl samples were injected onto a TSK G1000 PW column and eluted at 0.6 ml/min in 0.1 M trisodium phosphate buffer (pH 8.0) for analysis of MDA as described by Csallany et al. (18). Detection of released MDA was accomplished by using the Beckman variable wavelength detector at 267 nm and was compared with the nonhydrolyzed samples for quantitation purposes (18). This detector allowed the simultaneous reading of absorbance at two wavelengths (267 nm and 250 nm) and the scanning (220 nm to 300 nm in 5-nm increments) of eluting peaks without stopping the flow of the mobile phase. Compensation is automatically made in the resulting spectrum for the change in concentration of the peak material.

MDA co-chromatography and derivatization. The MDA peak from TSK G1000 PW chromatography was collected and co-chromatographed with standard MDA on this same column. Preparation of standard MDA is described by Csallany et al. (18). The absorbance ratio at 267 nm: 250 nm was monitored, and scans for 200 nm to 300 nm were measured during the run using the Beckman Model 165 detector. Derivatization of collected MDA peaks with 4,4′-sulfonyldianiline was accomplished as described by Csallany et al. (18). The relative fluorescence of all samples was determined at 470 nm/540 nm, and appropriate fluorescence spectra were done.

Protein determination. Two spectral methods were used to determine protein content of the nonhydrolyzed samples. The Warburg and Christian method (19) utilizes the absorbance at 260 nm and at 280 nm (mg protein/ml = $1.55 \times A_{280} - 0.775 \times A_{260}$). The Murphy and Kies method (20) uses the absorbance difference between 215 and 225 nm (mg protein/ml = $0.154 \times [A_{215} - A_{225}]$). All absorbance readings were obtained using the Beckman DU-8 Spectrophotometer (Beckman Instruments, Inc.).

RESULTS

The vitamin E-related, high molecular weight material from the Sephadex G-15 void volume was further separated on an HPLC size exclusion TSK G2000 SW column. Six proteins were observed, each having the same fluorescence characteristics (275 nm/350 nm) as the original void volume material. Attempts to separate this large protein on Sephadex G-50, G-100 and G-200 columns were unsatisfactory, although they did show the presence of more than one fluorescent compound in the G-15 column void volume material.

The water-soluble portion of the chloroform/methanol (2:1) extracts from livers of six vitamin E-deficient, and six vitamin E-supplemented (30 ppm) mice were injected onto the TSK G2000 column. One of the six proteins discussed above increased significantly due to vitamin E deficiency (Fig. 1). It had the smallest molecular weight of the six proteins, determined to be 20,000 daltons, and eluted at 10.33 ± 0.0125 ml.

These proteins were analyzed for the presence of MDA. Water extracts of mouse liver tissue were injected onto the TSK G2000 SW column, and the major peaks were collected including the 20,000 m.w. compound. Protein determinations were made, and the collected fractions were subjected to hydrolysis. Both the nonhydrolyzed and hydrolyzed fractions were chromatographed on the TSK

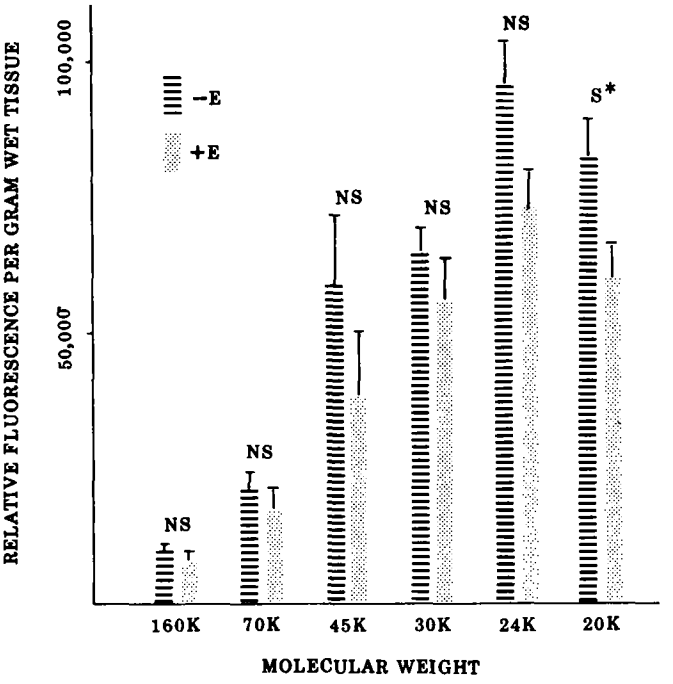

FIG. 1. Summary of the significant differences due to vitamin E seen in the protein materials separated from the Sephadex G-15 void volume peak. NS, not significant (Student's t-test); S*, significant ($P < .05$).

G1000 PW column for MDA analysis. Figure 2 shows a typical elution profile of these chromatographed fractions with the MDA peak eluting at 47.7 min. No MDA was found in any of the nonhydrolyzed fractions. The hydrolyzed 24,000 and 20,000 m.w. samples had the largest MDA peaks, which were 10- to 30-fold greater than those from other hydrolyzed samples. The absorbance ratio of 250 nm:267 nm was the same for all MDA peaks and equalled that obtained during a chromatographic run of standard MDA (0.37 ± .01). Scans from 220 nm to 300 nm were run during elution of each MDA peak. The same shape and maximum absorbance at 265 nm was observed for standard MDA and for each hydrolyzed fraction with a major MDA peak.

A six- to seven-month-old vitamin E-deficient mouse liver was found to contain about 900 ng of MDA per gram wet liver tissue before hydrolysis and 4000 ng of MDA per gram after hydrolysis.

The MDA peak from standard MDA and hydrolyzed samples was collected from the TSK G1000 PW column. An equally absorbant (267 nm) mixture of standard MDA and sample MDA was reapplied to the TSK G1000 PW column. The mixture eluted identically to the MDA standard (Fig. 3).

Derivatized sample MDA exhibited the same fluorescence as that seen for derivatized standard MDA (Fig. 4). The excitation maximum was 470 nm, and the emission maximum was 540 nm. The quantity of MDA present was determined by comparison of the fluorescence of the sample derivative at 470 nm/540 nm with that of the derivative of standard MDA and comparison of the MDA peak height to that of a standard curve of pure MDA chromatographed on the TSK G1000 PW column. These two quantities were almost equal.

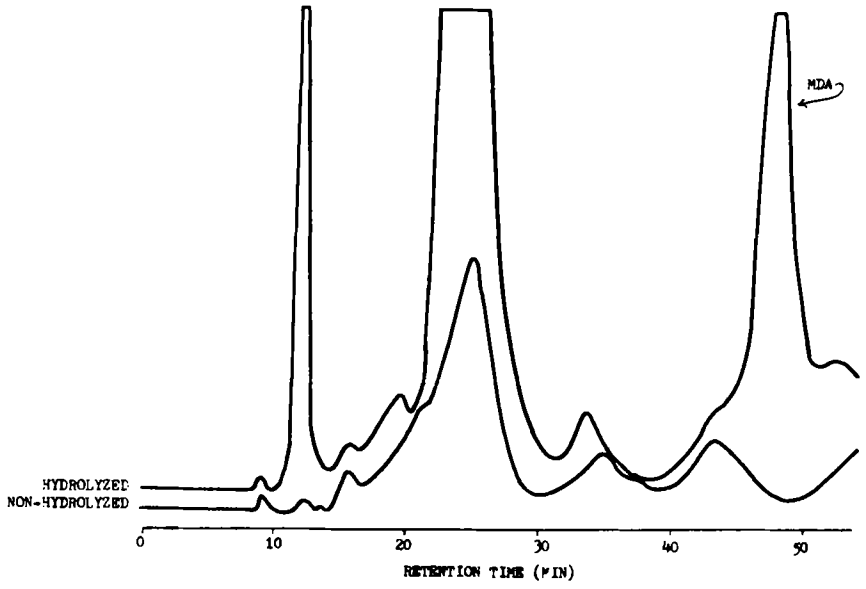

FIG. 2. Elution profile of hydrolyzed and nonhydrolyzed proteins (from G-15 void volume) chromatographed on a TSK-Gel 1000 PW column in 0.1 M phosphate buffer, pH 8.0, 0.6 ml/min.

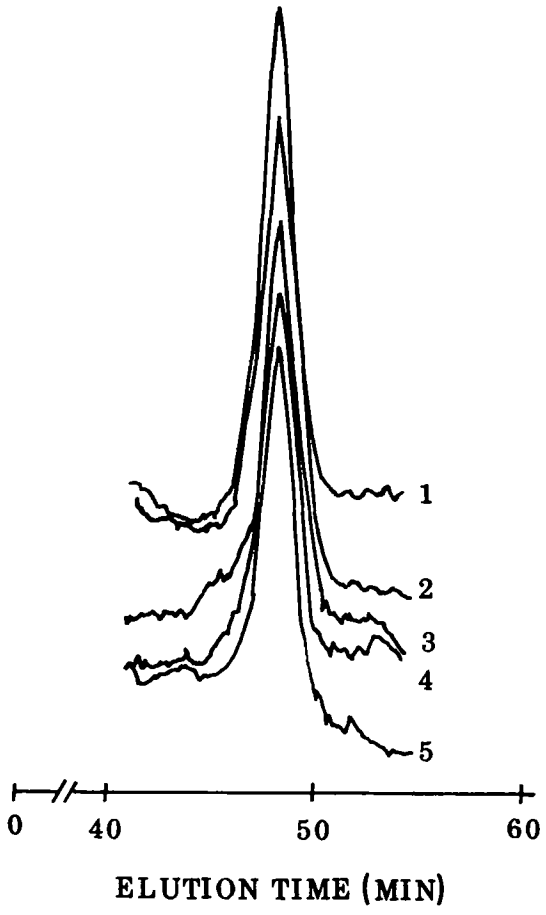

FIG. 3. Co-chromatography of collected MDA peak from hydrolyzed proteins (separated from Sephadex G-15 void volume) with standard MDA on a TSK-Gel 1000 PW column: (1) mixture of MDA + hydrolyzed (HD) 24,000 m.w. material (MDA + HD − 24,000), (2) HD − 24,000, (3) MDA + HD − 20,000, (4) HD − 20,000, and (5) MDA standard.

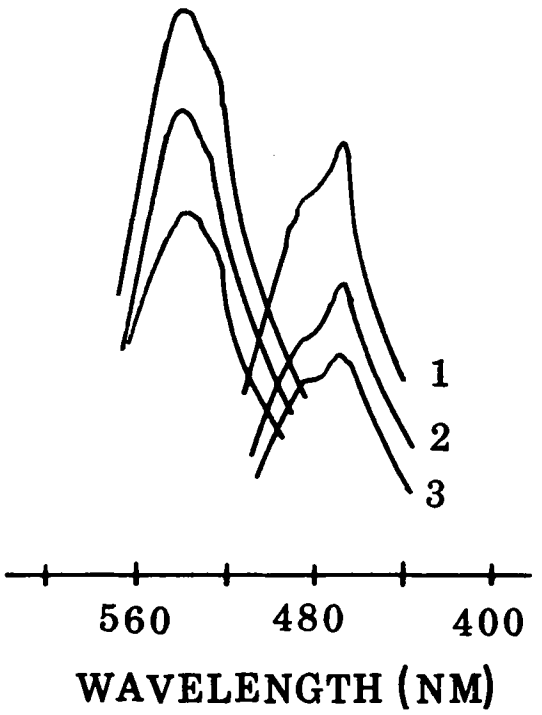

FIG. 4. Excitation and emission spectra of the fluorescent derivatives of the following: (1) malondialdehyde standard, and malondialdehyde released from: (2) 24,000 m.w. protein-like material, and (3) 20,000 m.w. protein.

A comparison of the concentration of MDA with respect to protein content is found in Table 1. Proteins less than 75,000 daltons all contained some MDA. The 20,000 and 24,000 m.w. proteins contained the largest amounts.

DISCUSSION

The water-soluble portion of chloroform/methanol (2:1) extracts was examined for the presence of fluorescent

TABLE 1

Malondialdehyde Content of Fluorescent Proteins in Water Extract of Mouse Liver

Material (m.w.)	μg MDA/ mg protein	Molecules MDA/ protein molecules
75,000	0[a]	0
45,000	3[b]	2
30,000	3[b]	1
24,000	11[b]	4
20,000	9[b]	3

[a]Average of two liver tissues.

[b]Average of three liver tissues.

compounds, as the magnitude of fluorescence from the organic solvent-soluble lipofuscin pigments does not account for the large amount of fluorescence observed microscopically in the liver. There is approximately 100 times more fluorescence in the water extract than in the organic solvent extract. This generally does not account for all of the fluorescence in the intact tissue. Any additional fluorescent materials is present in the insoluble interphase material removed during the extraction process.

Each of the materials separated by TSK G2000 SW chromatography of the Sephadex G-15 column void volume material had a molecular weight greater than 15,000 daltons and fluoresced near 350 nm when excited at 275 nm. From the above findings, it is apparent that each of these compounds are proteins. It generally is observed that in neutral solutions of tissue extracts, there are few materials other than proteins that fluoresce in the 340 nm–350 nm region when excited near 280 nm (21). Under these conditions, nucleic acids, purines and pyrimidines fail to show any fluorescence (21). MDA itself has no sizeable fluorescence, but Chio and Tappel (22) have reported that the formation of Schiff bases by the condensation of the carbonyl groups of MDA and the amine structure of amino acids form 1-amino-3-iminopropene (R-NH-CH = CH -CH = NR) products and have fluorescence in the 450 nm region when excited at 350 nm–360 nm. If this fluorescence was present in the protein materials studied above, it was at such a low level that it could not be detected.

Due to the low concentration of these protein materials within the cell and the necessary dilutions arising from the separation techniques, great difficulty was encountered in their separation. The TSK G2000 PW column was the largest size exclusion column available but still was limited in its ability to completely separate any concentrated samples. A large enough sample had to be used to allow detection and identification of released MDA, but concurrently the sample had to be small enough to allow adequate separation on the column. A compromise that allowed an approximation of the MDA content of these materials to be determined for the primary purpose of comparison between the separated peaks was made.

An approximation of the relative rate for the formation of the condensation product of a primary amino containing compound with carbonyl compounds is shown (Fig. 5)

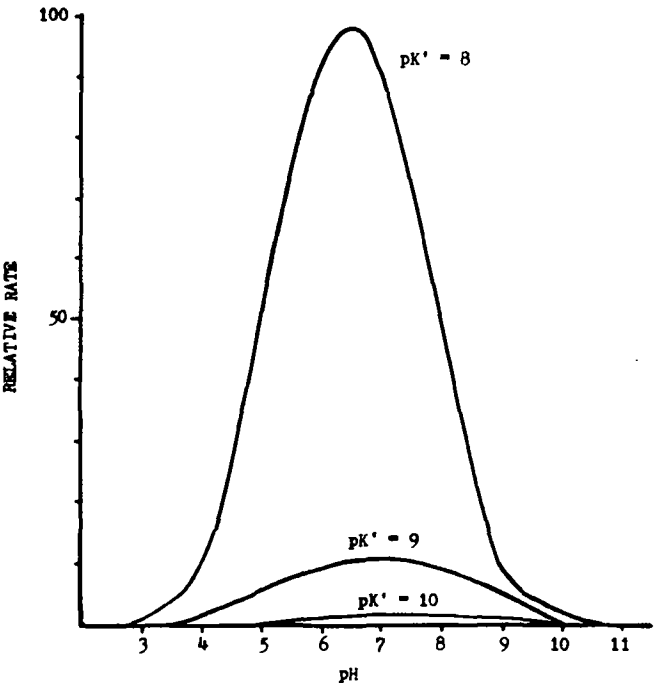

FIG. 5. Relative reaction rate for the formation of the Schiff base vs pH when the pK′ of the amine group is 8, 9 or 10.

vs pH when the pK′ of the amine group is either 8, 9 or 10. The reaction is dependent on the pH of the medium keeping both the carbonyl group protonated and the primary amino group deprotonated (neutral). This will occur at a pH midway between the pK′ for the carbonyl group and that for the amino group. The reversibility of this reaction was the basis for the hydrolysis procedure used to liberate MDA from the protein compounds separated on the TSK G2000 SW column. A high pH was chosen to guard from the possible production of new MDA by acid treatment (23).

Only one of the proteins was found to be significantly related to dietary vitamin E. All proteins that were smaller than 75,000 daltons were found to contain bound MDA with the smallest proteins containing the most MDA. It would be expected that vitamin E deficiency would lead to an increase in MDA levels in the cell and thus to an increase in protein-bound MDA. The 24,000 m.w. material, although not significantly increased ($p < 0.75$), tended to be elevated due to vitamin E deficiency. A larger number of samples might have shown the difference to be significant. It also is possible that within localized regions of the cell such as membranes, specific proteins that readily react with MDA may be present. If these proteins were present only in these areas and if MDA formation was nearly as great as that of the protein, it would not be expected to increase significantly due to vitamin E deficiency unless an increased production of the protein accompanied it.

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REFERENCES

1. Bourne, C.H. (1973) *Prog. Brain Res. 40,* 187-201.
2. Porta, E.A., and Hartroft, W.S. (1969) in *Pigments in Pathology* (Wolman, M., ed.), pp. 193-235, Academic Press, New York.
3. Brizzee, K.R., Harkin, J.C., Ordy, J.M., and Kaack, B. (1975) in *Aging* (Brody, H., Harman, D., and Ordy, J.M., eds.) Vol. I, pp. 39-78, Raven Press, New York.
4. Reichel, W. (1968) *J. Gerontol. 23,* 145-153.
5. Moore, T., and Wang, Y.L. (1974) *Br. J. Nutr. 1,* 53-64.
6. Reddy, K., Fletcher, B., Tappel, A., and Tappel, A.L. (1973) *J. Nutr. 103,* 908-915.
7. Weglicki, W.B., Reichel, W., and Nair, P.O. (1968) *J. Gerontol. 23,* 469-475.
8. Raychaudhuri, C., and Desai, I.E. (1971) *Science 173,* 1028-1029.
9. Sheldahl, J.A., and Tappel, A.L. (1974) *Exp. Gerontol. 9,* 33-41.
10. Strehler, B.L. (1964) in *Advances in Gerontological Research* (Strehler, B.L., ed.), pp. 343-384, Academic Press, New York.
11. Csallany, A. Saari, Ayaz, K.L., and Su, L.-C. (1977) *J. Nutr. 107,* 1792-1799.
12. Brizzee, K.R., Harkin, J.C., Ordy, J.M., and Kaack, B. (1975) in *Aging* (Brody, H., Harman, D., and Ordy, J.M., eds.) Vol. 1, pp. 39-78, Raven Press, New York.
13. Srebo, Z. (1966) *Naturwissenschaffen 53,* 590.
14. Trostler, N., Brady, P.S., and Romsos, D.R., and Leveille, G.A. (1979) *J. Nutr. 109,* 345-352.
15. Csallany, A. Saari, and Ayaz, K.L. (1976) *Lipids 11,* 412-417.
16. Manwaring, J.D., and Csallany, A. Saari (1981) *J. Nutr. 111,* 2172-2179.
17. Snedecor, G.W., and Cochran, W.G. (1967) in *Statistical Methods* (Snedecor, W.W., and Cochran, W.G., eds.) pp. 59-60, Iowa State University Press, Ames, Iowa.
18. Csallany, A. Saari, Guan, M.D., Manwaring, J.D., and Addis, P.B. (1984) *Anal. Biochem. 142,* 277-283.
19. Warburg, O., and Christian, W. (1941) *Biochem. Z. 310,* 384-421.
20. Murphy, J.B., and Kies, M.W. (1960) *Biochim. Biophys. Acta 45,* 382-384.
21. Udenfriend, S. (1962) in *Fluorescence Assay in Biology and Medicine* (Horecker, B., Kaplan, N.O., Marmur, J., and Scheraga, H.A., eds.), Vol. I, pp. 201-207, Academic Press, New York.
22. Chio, K.S., and Tappel, A.L. (1969) *Biochemistry 8,* 2821-2827.
23. Pryor, W.A., Stanley, J.P., and Blair, E. (1976) *Lipids 11,* 370-379.

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Effect of Diethyl Ether on Phosphatidylcholine Biosynthesis in Hamster Organs

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The effect of diethyl ether anesthesia on phosphatidylcholine biosynthesis in hamster organs was investigated. Ether administration did not affect the incorporation of radioactive choline into phosphatidylcholine in the liver, heart, lung, brain and spleen. A significant (29%) decrease in the labeling of phosphatidylcholine was detected in the kidney of ether-treated hamsters. Reduction in phosphatidylcholine labeling was not due to a diminished radioactive choline uptake but a decrease in the conversion of phosphocholine to CDP-choline. The accumulation of labeled phosphocholine was caused by the translocation of CTP:phosphocholine cytidylyltransferase from microsomal (more-active) form to cytosolic (less-active) form. Ether administration appears to modulate the cytidylyltransferase in hamster kidney differently than that in other hamster organs.
Lipids 23, 656–659 (1988).

Phosphatidylcholine is the principal phospholipid in mammalian tissues (1). The majority of phosphatidylcholine is synthesized via the CDP-choline pathway (2,3), and the remainder is formed either from the progressive methylation of phosphatidylethanolamine or from the exchange of the base group of an existing phospholipid with choline (3). In the CDP-choline pathway, choline initially is phosphorylated to phosphocholine by choline kinase (2,3). Phosphocholine is converted to CDP-choline by CTP: phosphocholine cytidylyltransferase (2,3). The CDP-choline formed is rapidly condensed with 1,2-diacylglycerol for the formation of phosphatidylcholine. The conversion of phosphocholine to CDP-choline is regarded as the rate-limiting step of this pathway (2–4).

The effect of aromatic anesthetics on phosphatidylcholine biosynthesis is well-documented (5,6). Administration of polychlorinated biphenyls reduced the incorporation of the labeled phosphate into the choline-containing phospholipids in rat liver, whereas phenobarbital enhanced such incorporation (5). In a subsequent study (6), these investigators demonstrated that the administration of phenobarbital resulted in substantial decreases of choline kinase and microsomal phosphocholine cytidylyltransferase activities. Choline phosphotransferase and cytosolic CTP:phosphocholine cytidylyltransferase activities were not significantly affected by such treatment.

Diethyl ether is a commonly administered anesthetic for small laboratory animals. Previously, we demonstrated that the labeling of phosphatidylethanolamine from ethanolamine was reduced in the kidney of the diethyl ether-anesthetized hamsters (7). In this study, the effect of diethyl ether on phosphatidylcholine biosynthesis in hamster organs was examined.

MATERIALS AND METHODS

Choline iodide, phosphorylcholine chloride and CDP-choline were obtained from Sigma Chemical Co. (St. Louis, MO). [Methyl-^{3}H]choline and CDP-[methyl-^{14}C]-choline were purchased from NEN division, DuPont Co. (Dorval, Quebec). Thin layer chromatographic plates (sil-G25) were the product of Brinkmann (Rexdale, Ontario). Phospho-[methyl-^{3}H]choline was synthesized enzymatically from [methyl-^{3}H]choline by the action of yeast choline kinase as described (8). All other chemicals were of reagent grade and were obtained through Fisher Scientific Co. (Winnipeg).

Male Syrian golden hamsters, 120 ± 10 g, were maintained on Purina hamster chow and tap water, ad libitum, in a light- and temperature-controlled room.

Diethyl ether was administered to the hamster by inhalation as described (7). The animal was placed in a 2-l sealed container, and a cotton ball containing 2 ml of diethyl ether was introduced into the container. Subsequent to the loss of the righting reflex (3–4 min), the animal was removed from the container and labeled choline (0.5 ml of a 20 mM choline chloride solution) containing 20 μCi was injected intraperitoneally. Light anesthesia was administered by the inhalation of a ether/air mixture for the next 60 min. A minimum respiratory rate of 40/min was maintained throughout the experiment. Identical doses of labeled choline were injected into the non-anesthetized animals, and these animals were used as controls. At the prescribed time interval, the control and the anesthetized animals were killed by decapitation, and the organs were rapidly removed and placed in ice-cooled saline (0.9% NaCl). The organs were weighed and homogenized in $CHCl_3/CH_3OH$ (2:1, v/v) to yield a 10% (w/v) homogenate. The homogenate was centrifuged, and the pellet was re-extracted twice with $CHCl_3/CH_3OH$ (2:1, v/v). The extracts were pooled, and an aliquot was taken for the determination of total uptake of radioactivity. Phase separation of the pooled extract was achieved by adding water to the extract until a $CHCl_3/CH_3OH/H_2O$ rato of 4:2:3 (v/v/v) was obtained. Radioactivity in the organic phase was determined, and phosphatidylcholine in this phase was analyzed by thin layer chromatography with a solvent containing $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (70:30:2:4, v/v/v/v) (4). Choline-containing metabolites in aqueous phase was analyzed by thin layer chromatography with a solvent containing CH_3OH/0.6% NaCl/NH_4OH (50:50:5, v/v/v) (4). Because a substantial amount of choline was oxidized into betaine in the liver (2), the labeling of betaine also was determined. Betaine was separated from CDP-choline after thin layer chromatography by a Norit A charcocal column (0.5 × 3 cm) equilibrated with 2% ethanol. Betaine was eluted from the column by 20 ml 2% ethanol, whereas CDP-choline was eluted by subsequent addition of 10 ml 40% ethanol containing 1% NH_4OH.

Subcellular fractions of the hamster organs were prepared by differential centrifugation as described (4). Choline kinase in the cytosolic fraction was assayed with labeled choline (9). CTP:phosphocholine cytidylyltransferases in the cytosolic and microsomal fractions were assayed with labeled phosphocholine at exactly four hr

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after the tissues were homogenized (10). In addition, cytosolic CTP:phosphocholine cytidylyltransferase activity was determined in the presence of total liver phospholipid extract (2 mg/ml) and 0.1 mM oleate. Maximal stimulation of the cytosolic cytidylyltransferase was obtained with these lipids (11). Microsomal CDP-choline: 1,2-diacylglycerol choline phosphotransferase was assayed with labeled CDP-choline in the presence of Tween-20 and exogenous diacylglycerol (12). Protein was determined by the procedure of Lowry et al. (13), and lipid phosphorus content was analyzed by the method of Bartlett (14).

Radioactivity was determined by liquid scintillation counting using channels' ratio calibration method. Student's t-test was used for statistical analysis of the results. The level of significance was $P < 0.05$.

RESULTS

The effect of diethyl ether on the labeling of phosphatidylcholine in hamster organs was investigated in a time-course study. Ether-anesthetized hamsters were injected intraperitoneally with labeled choline. Unanesthetized hamsters were used as controls. The animals were killed at the prescribed time intervals, and the phosphatidylcholine content and labeling in the organs were determined. Ether anesthesia did not affect the amount of phosphatidylcholine (estimated by μmol of lipid-P/g tissue wet wt.) in all hamster organs at 30 and 60 min of treatment. However, at 30 min of anesthetic treatment, a 12% decrease ($0.05 < P < 0.1$) in phosphatidylcholine labeling was observed in the kidney. At 60 min, this decrease was more prominent (29%). No significant change in labeling of phosphatidylcholine was detected in other organs (Table 1). One factor that might cause the reduction of phosphatidylcholine labeling in the kidney was a decrease in the uptake of labeled choline. Hence, total labeled choline uptake in hamster organs at 60 min of treatment was determined (Table 2). The total ratioactivity taken up by the kidney was similar between the control and anesthetized hamsters. Although the total choline uptake in the liver might be slightly increased during anesthesia, such differences were not statistically significant.

TABLE 1

Incorporation of Radioactivity into Phosphatidylcholine in Hamster Organs During Diethyl Ether Anesthesia[a]

Tissue	Control dpm/g wet wt. ($\times 10^{-5}$)	Diethyl ether-treated dpm/g wet wt. ($\times 10^{-5}$)
Heart	1.25 ± 0.45 (8)	0.95 ± 0.37 (8)
Liver	8.27 ± 1.36 (10)	9.58 ± 1.80 (10)
Kidney	8.19 ± 2.20 (8)	5.84 ± 1.36* (8)
Lung	7.58 ± 1.74 (5)	6.22 ± 0.50 (5)
Brain	0.31 ± 0.04 (5)	0.32 ± 0.04 (5)
Spleen	2.77 ± 0.44 (4)	2.37 ± 0.65 (3)

*$P < 0.05$.

[a][Methyl-^{3}H]choline was injected intraperitoneally into control and diethyl ether anesthetized hamsters as described in Methods. After 60 min, hamsters were decapitated, and the organs were removed and homogenized in chloroform/methanol (2:1, v/v). The homogenates were separated into aqueous and organic phases by the addition of chloroform and water. Phosphatidylcholine in the organic phase was isolated by thin layer chromatography. The results are depicted as mean ± standard deviation (number of experiments).

TABLE 2

Total Uptake of [Methyl-^{3}H]Choline by Hamster Organs[a]

Tissue	Control dpm/g wet wt. ($\times 10^{-5}$)	Diethyl ether-treated dpm/g wet wt. ($\times 10^{-5}$)
Heart	4.64 ± 1.71 (8)	4.14 ± 1.01 (8)
Liver	24.34 ± 4.17 (10)	27.64 ± 2.92 (10)
Kidney	18.75 ± 4.64 (8)	18.56 ± 4.52 (8)
Lung	11.68 ± 2.47 (5)	12.01 ± 4.30 (5)
Brain	1.87 ± 0.55 (5)	1.40 ± 0.15 (5)
Spleen	9.07 ± 2.53 (4)	8.58 ± 1.14 (4)

[a][Methyl-^{3}H]choline was injected intraperitoneally into control and diethyl ether anesthetized hamsters as described in Methods. After 60 min, hamsters were decapitated, and the organs were removed and homogenized in chloroform/methanol (2:1, v/v). An aliquot was taken for radioactivity determination. The results are depicted as mean ± standard deviation (number of experiments).

The labeling of the choline-containing metabolites in the CDP-choline pathway in the kidney at 60 min of treatment also was determined. As depicted in Table 3, labeling of phosphocholine was substantially increased (41%). Such increase suggests that the ability to convert phosphocholine into CDP-choline was impaired in the kidney during ether anesthesia. Because the uptake of labeled choline in the liver also might be perturbed by ether anesthesia (Table 2), the labeling of the hepatic choline-containing metabolies also was determined. No significant difference was detected between the two animal groups (Table 3). In addition, labeling of betaine was similar between the control and experimental animals (data not shown). These results confirm that total choline uptake in the liver was not changed between the control and ether-treated animals.

The accumulation of radioactivity in the phosphocholine fraction in the kidney of the anesthetized hamster might be caused by a change in the activity of the enzymes in the CDP-choline pathway. Thus, the activities of these enzymes in the hamster organs were determined after 60 min of ether administration. In the kidney, no change in either choline kinase or choline phosphotransferase activities was detected between the two animal groups (Table 4). Interestingly, CTP:phosphocholine cytidylyltransferase activity in the microsomal fraction was substantially reduced in the anesthetized animal, whereas the enzyme activity in the cytosol was not changed. However, in the presence of lipid activators a significant increase in cytosolic enzyme activity was observed in the anesthetized kidney (Table 4). Total cytidylyltransferase activity (μmol/min/g tissue wet wt.) was calculated from the sum of the microsomal and cytosolic enzyme activities. The total enzyme activity was reduced in the kidney of the anesthetized animal in the absence of exogenous lipid activators in the cytosol. When the cytosolic enzyme was fully activated, there was no difference in total enzyme activity. As an additional control, the enzymes of the CDP-choline pathway also were assayed in the other organs. No significant differences in any of the enzyme activities were detected between the control and anesthetized animals (data not shown).

TABLE 3

Radioactivity Incorporated into Choline-containing Metabolites in Hamster Organs[a]

Metabolites	Kidney		Liver	
	Control dpm/g wet wt. ($\times 10^{-5}$)	Diethyl ether-treated dpm/g wet wt. ($\times 10^{-5}$)	Control dpm/g wet wt. ($\times 10^{-5}$)	Diethyl ether-treated dpm/g wet wt. ($\times 10^{-5}$)
Choline	0.34 ± 0.23 (8)	0.30 ± 0.18 (8)	0.15 ± 0.02 (4)	0.17 ± 0.03 (3)
Phosphocholine	1.45 ± 0.56 (8)	2.05 ± 0.17* (8)	1.51 ± 0.46 (4)	1.46 ± 0.24 (3)
CDP-Choline	0.32 ± 0.25 (8)	0.56 ± 0.30 (8)	0.02 ± 0.01 (4)	0.02 ± 0.01 (3)

*$P < 0.05$.

[a]Tissue homogenates were separated into aqueous and organic phase by the addition of chloroform and water as described in Methods. The aqueous phase was analyzed for labeled metabolites of the CDP-choline pathway. The results are depicted as mean ± standard deviation (number of experiments).

TABLE 4

The Activities of Phosphatidylcholine Biosynthetic Enzymes from Hamster Kidney[a]

Enzymes	Specific activity	
	Control nmol/min/mg protein	Diethyl ether-treated nmol/min/mg protein
Choline kinase		
(Cytosolic)	1.05 ± 0.05 (4)	1.04 ± 0.05 (4)
Phosphocholine cytidylyltransferase		
(Microsomal)	3.37 ± 0.78 (4)	1.71 ± 0.57* (3)
(Cytosolic)	0.66 ± 0.29 (4)	0.62 ± 0.24 (4)
(Cytosolic + lipids)	2.07 ± 0.22 (4)	2.65 ± 0.27* (4)
Choline phosphotransferase		
(Microsomal)	8.11 ± 0.66 (4)	8.33 ± 1.10 (4)

*$P < 0.05$.

[a]Kidneys were obtained from control and diethyl ether-anesthetized hamsters. Subcellular fractions from various organs were prepared as described in Methods. These fractions were assayed for phosphatidylcholine-synthesizing enzyme activities. The results are depicted as mean ± standard deviation (number of experiments).

DISCUSSION

In this study, the effect of diethyl ether anesthesia on phosphatidylcholine biosynthesis via the CDP-choline pathway in hamster organs was investigated. Our results clearly demonstrated that such treatment did not produce any effect on phosphatidylcholine biosynthesis in hamster organs other than the kidney. The reduction in phosphatidylcholine biosynthesis in hamster kidney under ether anesthesia (29%) was quantitatively similar to that reported for phosphatidylethanolamine biosynthesis (30% reduction) (7). The reduced labeling of phosphatidylcholine was not caused by a decrease in labeled choline uptake but probably resulted from a reduction in the conversion of phosphocholine to CDP-choline. Such conversion, which was catalyzed by CTP:phosphocholine cytidylyltransferase, has been regarded as the rate limiting step in the CDP-choline pathway (2–4). Cytidylyltransferase activity is located in both the microsomal and cytosolic fractions (3). The enzyme in the cytosolic fraction is enhanced upon storage at 4 C or at room temperature (10). To obtain valid comparisons, all cytidylyltransferase assays in the cytosol were performed exactly four hr after tissue homogenization (4). Because the cytosolic enzyme has a lipid requirement (2,10,11), lipids were added to the assay mixture to obtain maximum enzyme activation.

Current evidence suggests that the microsomal form of the enzyme is the active form responsible for the conversion of phosphocholine to CDP-choline in vivo (2, 15–17) and the translocation of the cytidylyltransferase from one subcellular fraction to another is an important mechanism for the overall regulation of phosphatidylcholine biosynthesis (2,18). Because the total activity in microsomal form was reduced with a corresponding increase in the cytosolic form (when fully activated), together with the fact that the combined activities in these two forms were not changed, we postulate that ether treatment promotes the translocation of the kidney cytidylyltransferase from the microsomal form to the cytosolic form. Such translocation would cause a reduction in the conversion of phosphocholine to CDP-choline. This was confirmed by the analysis of choline-containing metabolites, and also by the observation that the total cytosolic enzyme activity was not increased without the presence of exogenous lipids. Indeed, the combined

enzyme activity in the two forms when assayed without exogenous lipid was significantly decreased in the anesthetized kidney. The transfer of the microsomal cytidylyltransferase to the cytosolic fraction during ether anesthesia would provide the kidney with a facile mechanism to attenuate the rate of phosphatidylcholine biosynthesis.

One intriguing aspect of this study is the differential effect of diethyl ether on the translocation of the cytidylyltransferase in the kidney, but not in other hamster organs. Presently, only the cytidylyltransferase from the liver has been purified and studied in detail (19). Our results suggest that the enzyme in hamster kidney may be modulated differently than those found in other hamster tissues.

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REFERENCES

1. White, D.A. (1973) in *Form and Function of Phospholipids* (Ansell, G.B., Hawthorne, J.N., and Dawson, R.M.C., eds.) pp. 441-482, Elsevier Scientific Publishing Co., Amsterdam.
2. Pelech, S.L., and Vance, D.E. (1984) *Biochim. Biophys. Acta 779*, 217-251.
3. Vance, D.E., and Choy, P.C. (1979) *Trends Biochem. Sci. 4*, 145-148.
4. Zelinski, T.A., Savard, J.D., Man, R.Y.K., and Choy, P.C. (1980) *J. Biol. Chem. 255*, 11423-11428.
5. Ishidate, K., and Nakazawa, Y. (1976) *Biochem. Pharmacol. 25*, 1255-1260.
6. Ishidate, K., Tsuruoka, M., and Nakazawa, Y. (1980) *Biochim. Biophys. Acta 620*, 49-58.
7. Tam, S.W., and Choy, P.C. (1983) *Biochim. Biophys. Acta 754*, 111-115.
8. Paddon, H.B., and Vance, D.E. (1977) *Biochim. Biophys. Acta 488*, 181-189.
9. Choy, P.C. (1982) *J. Biol. Chem. 257*, 10928-10933.
10. Vance, D.E., Pelech, S.D., and Choy, P.C. (1981) *Methods Enzymol. 68*, 576-581.
11. Cornell, R., and Vance, D.E. (1987) *Biochim. Biophys. Acta 919*, 26-36.
12. Arthur, G., and Choy, P.C. (1984) *Biochim. Biophys. Acta 795*, 221-229.
13. Lowry, O.H., Roseborough, M.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265-275.
14. Bartlett, G.R. (1959) *J. Biol. Chem. 234*, 466-468.
15. Sleight, R., and Kent, C. (1980) *J. Biol. Chem. 255*, 10644-10650.
16. Sleight, R., and Kent, C. (1983) *J. Biol. Chem. 258*, 831-835.
17. Pelech, S.D., Cook, H.W., Paddon, H.B., and Vance, D.E. (1984) *Biochim. Biophys. Acta 795*, 433-440.
18. Vance, D.E., and Pelech, S.L. (1984) *Trends Biochem. Sci. 9*, 17-20.
19. Feldman, D.A., and Weinhold, P.A. (1987) *J. Biol. Chem. 262*, 9075-9081.

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Regulation of the Metabolism of Linoleic Acid to Arachidonic Acid in Rat Hepatocytes

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When 5×10^6 hepatocytes were incubated for 40 min with from 0.15 to 0.60 mM [1-^{14}C]linoleic acid, [1-^{14}C]6,9,12-octadecatrienoic acid, or [1-^{14}C]8,11,14-eicosatrienoic acid, there was a concentration-dependent acylation of radioactive metabolites into both triglycerides and phospholipids. When the concentration of either [1-^{14}C]linoleic acid or [1-^{14}C]8,11,14-eicosatrienoic acid exceeded 0.3 mM, there was no further increase in the metabolism of either fatty acid to other (n-6) metabolites. When the concentration of [1-^{14}C]6,9,12-octadecatrienoic acid exceeded 0.15 mM, there was an apparent substrate-induced inhibition in its metabolism to 8,11,14-eicosatrienoic acid. With all three substrates (0.3 mM), there was time-dependent metabolism to other (n-6) acids. Cells then were incubated simultaneously with 0.3 mM [1-^{14}C]linoleic acid along with 0.15 to 0.45 mM 6,9,12-octadecatrienoic acid or 8,11,14-eicosatrienoic acid. These exogenous nonradioactive (n-6) acids suppressed but did not abolish the conversion of [1-^{14}C]linoleate to radioactive arachidonate. These findings suggest that some linoleate is converted to arachidonate without intracellular mixing of 6,8,12-octadecatrienoic or 8,11,14-eicosatrienoic acids. This hypothesis is supported by the finding that exogenous linoleate did not markedly affect the metabolism of [1-^{14}C]6,9,12-octadecatrienoic or [1-^{14}C]8,11,14-eicosatrienoic acid by microsomal chain elongating or desaturating enzymes.
***Lipids 23,* 660–665 (1988).**

It is well-established that dietary linoleate is converted to arachidonate in the liver only by an alternating series of position-specific desaturases and malonyl-CoA-dependent chain elongation steps as follows: 18:2 → 18:3 → 20:3 → 20:4 (1). Liver lipids contain large amounts of linoleate and arachidonate but only low levels of 6,9,12-18:3 and 8,11,14-20:3, even though they are both obligatory intermediates in the synthesis of arachidonate. Several studies with liver microsomes (2–4) as well as with hepatocytes (5) have shown that desaturation of linoleate, by the 6-desaturase, is rate-limiting in this metabolic pathway. Although rate studies with microsomes define how rapidly various acids are metabolized, they have not been very informative in explaining why 6,9,12-18:3 and 8,11,14-20:3 are not found in significant amounts in liver lipids. When the CoA derivatives of 6,9,12-18:3 or 8,11,14-20:3 were incubated with lysophospholipids and liver microsomes, both substrates were readily acylated (6). Clearly, rate studies by themselves fail to explain the types of fatty acids found in liver phospholipids.

Recent studies by Christophersen and his colleagues (5,7,8) have shown that hepatocytes can be used effectively to study integrated aspects of polyunsaturated fatty acid desaturation and chain elongation along with the simultaneous acylation of metabolites into lipids. Thus, we carried out studies with hepatocytes from chow-fed rats to define if some linoleate was converted to arachidonate without intracellular mixing of the two obligatory intermediates required for converting dietary linoleate to arachidonate (9).

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Abbreviations: PL, phospholipids; TG, triglycerides; HPLC, high performance liquid chromatography.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Linoleic acid (52 Ci/mol) and [1-^{14}C]-8,11,14-eicosatrienoic acid (55 Ci/mol) were purchased from New England Nuclear (Boston, MA). [1-^{14}C]6,9,12-Octadecatrienoic acid (43 Ci/mol) was made by total synthesis as described (10). Other unsaturated fatty acids were obtained from Nu-Chek Prep. (Elysian, MN).

All solvents were either reagent or high performance liquid chromatography (HPLC) grade. Collagenase was purchased from Boehringer Mannheim GmbH (Indianapolis, IN).

Hepatocyte isolation. Male Sprague-Dawley rats (200–300 g) were maintained on a chow diet for at least one wk. Hepatocytes were isolated according to the general method of Seglen (11) as described (12). The cells were suspended at 5×10^6/ml in a buffer containing 113 mM NaCl, 4.5 mM KCl, 30 mM HEPES, 10 mM glucose, 1.1 mM $CaCl_2$, 1.3 mM KH_2PO_4, 26 mM $NaHCO_3$, 1.5 mM $MgCl_2$ and 1% fatty acid-free bovine serum albumin. Cell viability was routinely greater than 90% as determined by trypan blue exclusion.

Incubations. Hepatocytes, 5×10^6 in 2 ml of suspension buffer containing 10 mM lactic acid, were incubated at 37 C in a Dubnoff metabolic shaker under 95% oxygen – 5% carbon dioxide at a flow of 6 l/min. Incubations were initiated by adding the sodium salt of the fatty acid. The fatty acid concentrations are in the legends to the figures and tables.

Lipid isolation and analysis. Incubations were stopped by addition of 0.2 ml of 2 M formic acid and 2 ml of $CHCl_3$/MeOH (2:1, v/v). The contents of the vials were transferred to screw cap tubes, and the incubation vessels were washed twice with 4 ml of $CHCl_3$/MeOH (2:1, v/v), which then was combined with the original extract. The tubes were vortexed and centrifuged to obtain phase separation. A 50-μl aliquot of the upper aqueous layer was removed and counted in Formula-963 (New England Nuclear, Boston, MA) to quantitate the metabolism of fatty acids into water-soluble products. The bottom organic phase was taken to dryness under a stream of N_2. The lipids were dissolved in $CHCl_3$ and applied to a column of Unisil (Clarkson Chemical Co., Williamsport, PA) in a Pasteur pipette. Neutral lipids were recovered by eluting with 10 ml of $CHCl_3$, while phospholipids were recovered by eluting with 10 ml of methanol. The solvents were removed under N_2, and each lipid fraction was dissolved in 1 ml of $CHCl_3$/MeOH (2:1, v/v). Aliquots of 10 μl were removed from the phospholipid fraction to determine the total nmol of fatty acids incorporated into polar lipids. The remaining phospholipids were saponified by stirring overnight with 1 N KOH in ethanol/H_2O (9:1, v/v).

Aliquots of 50 μl were removed from the neutral lipid fraction and applied to one lane of a preabsorbant zone of a Whatman LK6D plate (Whatman, Clifton, NJ), while another 200 μl was applied to the preabsorbant zone of two other lanes. Thin layer chromatography was carried out using hexane/diethyl ether/acetic acid (80:20:2, v/v/v). The plates were scanned with a Packard Model 7220 radiochromatogram scanner. The free fatty acids and triacylglycerols from the single lane were scraped into scintillation vials and counted. The triacylglycerols from the other two zones were scraped into screw cap vials, and the lipids were saponified.

After saponification, the fatty acids were dissolved in 70 μl of acetonitrile. They were separated by reverse-phase HPLC using a Beckman HPLC consisting of two 112 pumps, a 420 controller and a model 1305 Bio-Rad variable wavelength detector set at 205 nm. Chromatography was carried out at room temperature using a 0.46 × 25 cm Zorbax ODS column (DuPont, Wilmington, DE). The solvent system was acetonitrile/water (80:20, v/v), in which the water was adjusted to pH 2.5 with phosphoric acid. The flow rate was 1 ml/min. Radioactivity was quantitated with a flow-through detector (Radiomatic Instruments and Chemical Co., Tampa, FL). The flow rate of ScintiVerse (Fisher Scientific Co., Cincinnati, OH) was 3 ml/min.

RESULTS

Figure 1 (bottom) shows that when increasing concentrations of [1-^{14}C]linoleate were incubated for 40 min with hepatocytes, there was an almost linear increase in the acylation of radioactive metabolites into phospholipids and triglycerides as well as metabolism into water soluble products. The triglycerides (TG) and phospholipids (PL) then were saponified and the fatty acids were analyzed by reverse-phase HPLC to define the composition of the radioactive (n-6) fatty acids. As shown in the upper panels of Figure 1, small amounts of linoleate were metabolized to other (n-6) acids and incorporated into both PL and TG. Although substrate saturation had not been achieved relative to overall acylation (Fig. 1, bottom), the results in the upper two panels show that maximum metabolism of the linoleate to other (n-6) acids occurred when the substrate concentration was about 0.3 mM. Hepatocytes were then incubated for various periods of time with 0.3 mM [1-^{14}C]linoleate. The results in Figure 2 (bottom) show that there is time-dependent

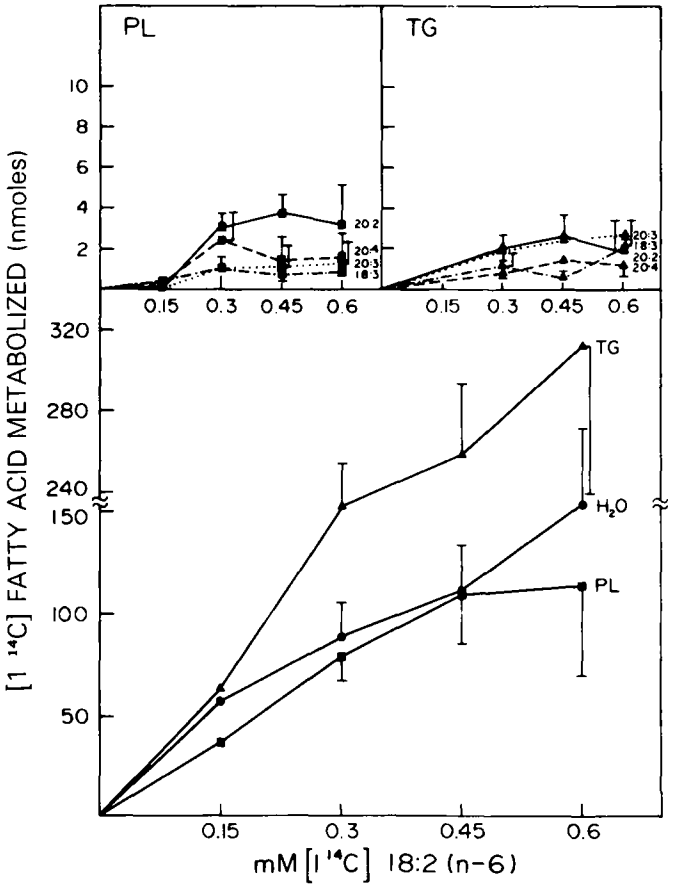

Fig. 1. Substrate-dependent metabolism of [1-^{14}C]linoleate (5 Ci/mol). Hepatocytes (5 × 10^6/2 ml) were incubated for 40 min. The total nmol of fatty acids esterified into phospholipids (PL), triglycerides (TG) or oxidized into water-soluble products (H_2O) are shown in the bottom panel. The upper panels show the nmol of (n-6) acids produced from [1-^{14}C]linoleate that are incorporated into PL and TG. Results are the averages ± the standard deviation of at least six experiments.

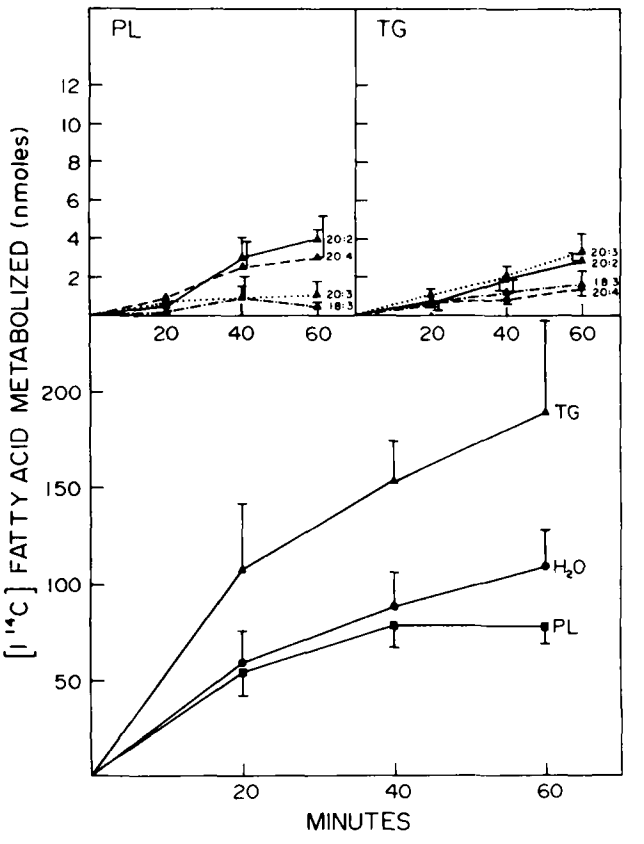

FIG. 2. Time-dependent metabolism of 0.3 mM [1-^{14}C]linoleate. The incubation conditions are as described in Figure 1. The total nmol of fatty acids esterified into water-soluble products (H_2O) are shown in the bottom panel. The upper panels show the nmol of (n-6) acids produced from [1-^{14}C]linoleate that are incorporated into PL and TG. Results are the averages ± the standard deviations of at least six experiments.

acylation of metabolites into both TG and PL as well as metabolism into water-soluble metabolites. The upper two panels of Figure 2 show that there is also time-dependent metabolism of linoleate to other (n-6) acids followed by their incorporation into both PL and TG.

Figures 3 and 4 show the results when an identical series of experiments were carried out using [1-^{14}C]-6,9,12-18:3 as substrate. The results (Fig. 3, bottom) show that there is substrate-dependent acylation of radioactive metabolites into PL and TG as well as metabolism into water-soluble products. However, when the distribution of radioactive (n-6) acids was measured in PL and TG, there was an apparent substrate-induced inhibition in the metabolism of 6,9,12-18:3 to 20-carbon (n-6) acids (Figure 3, top panels). When hepatocytes were incubated with 0.3 mM [1-^{14}C]6,9,12-18:3, there was time-dependent incorporation of metabolites into lipids (Fig. 4, bottom) as well as metabolism to other (n-6) acids. It is apparent that the metabolism of [1-^{14}C]6,9,12-18:3 to 20-carbon (n-6) acids followed by their acylation into phospholipids (Figure 4, top panel) increased in a nonlinear manner with time. These findings suggest that when the intracellular concentration of 6,9,12-18:3 is high, the substrate is an inhibitor of its own chain elongation to 8,11,14-20:3. Over time, there was rapid removal of substrate. The nmol of 6,9,12-18:3 remaining at 20, 40 and 60 min were, respectively 149 ± 29, 29 ± 22 and 25 ± 27. These results suggest that the inhibition of 6,9,12-18:3 chain elongation is a reversible process and that it is regulated by the intracellular concentration of 6,9,12-18:3 or its CoA derivative. This interpretation is consistent with the finding (Fig. 4, top) that the apparent rate of metabolism to 8,11,14-20:3 and to 5,8,11,14-20:4 increased in a nonlinear manner with time.

Figures 5 and 6 show how [1-^{14}C]8,11,14-20:3 was metabolized under identical conditions used for [1-^{14}C]-linoleate and [1-^{14}C]6,9,12-18:3. It is apparent from these results as well as of those of Hagve and Christophersen (8) that 8,11,14-20:3 is a much better substrate for desaturation than is linoleate.

The preceding studies compare how individual exogenous (n-6) acids were metabolized by hepatocytes. Subsequent studies thus were carried out to define if linoleate in part was metabolized directly to 5,8,11,14-20:4 in such a way that some of the radioactive 6,9,12-18:3 and 8,11,14-20:3 produced from [1-^{14}C]linoleate was not available for acylation into hepatocyte lipids. The results in Table 1 compare the metabolism of 0.3 mM [1-^{14}C]linoleate when the incubations also contained increasing amounts of nonradioactive 6,9,12-18:3 and 8,11,14-20:3. These results show that the addition of the two exogenous nonradioactive (n-6) acids did not markedly influence the incorporation of radioactive metabolites into either TG or PL. Under these conditions, the 6,9,12-18:3 and 8,11,14-20:3 were themselves readily incorporated into both TG and PL (subsequent results). These findings simply show

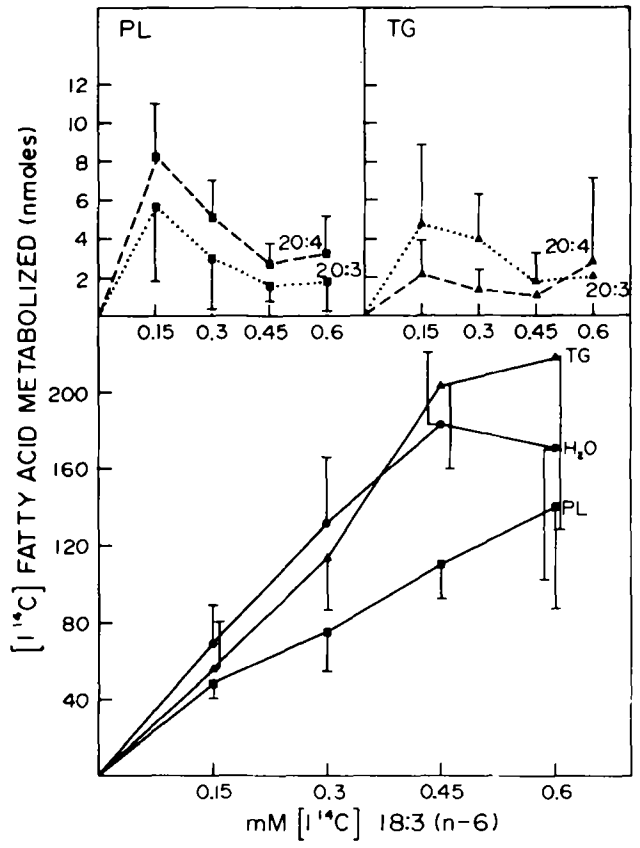

FIG. 3. Substrate-dependent metabolism of 0.3 mM [1-^{14}C]6,9,12-octadecatrienoic acid (2 Ci/mol). The conditions are as described in Figure 1. Results are the average ± the standard deviations of four experiments.

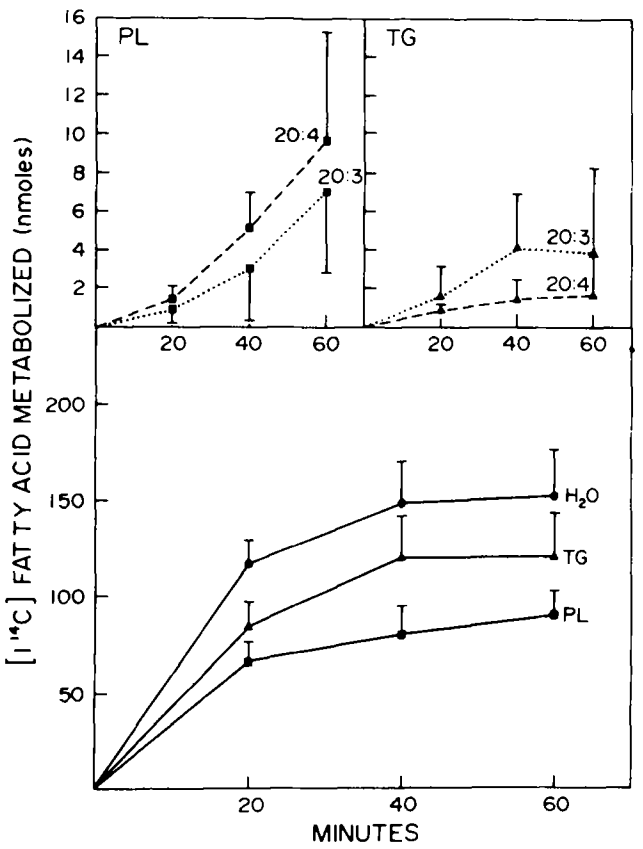

FIG. 4. Time-dependent metabolism of [1-^{14}C]6,9,12-octadecatrienoic acid (2 Ci/mol). The conditions are as described in Figure 2. Results are the average ± the range of two experiments.

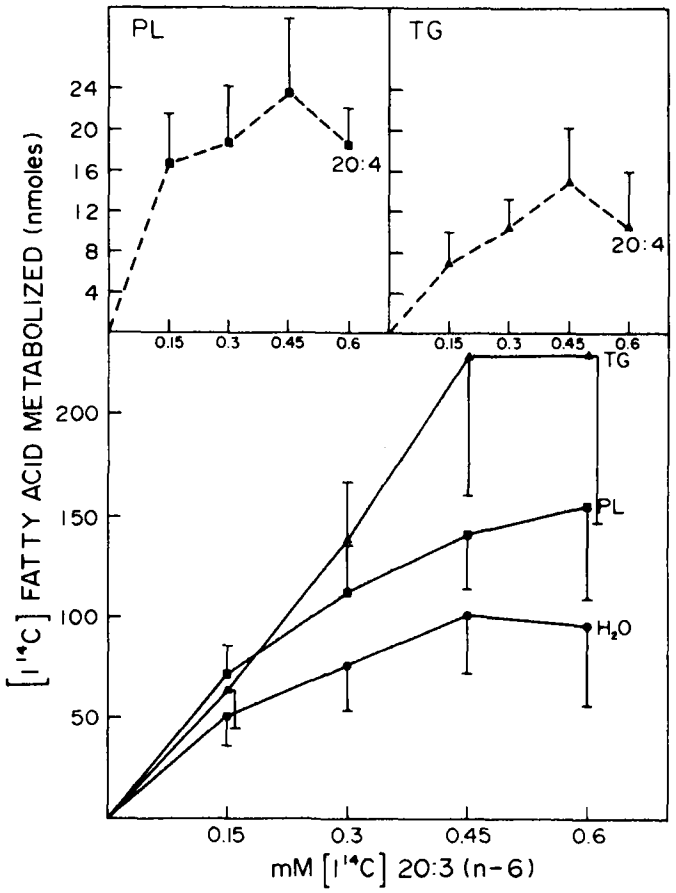

FIG. 5. Substrate-dependent metabolism of [1-^{14}C]8,11,14-eicosatrienoic acid (2, Ci/mol). The conditions are as described in Figure 2. Results are the average ± the standard deviation of five experiments.

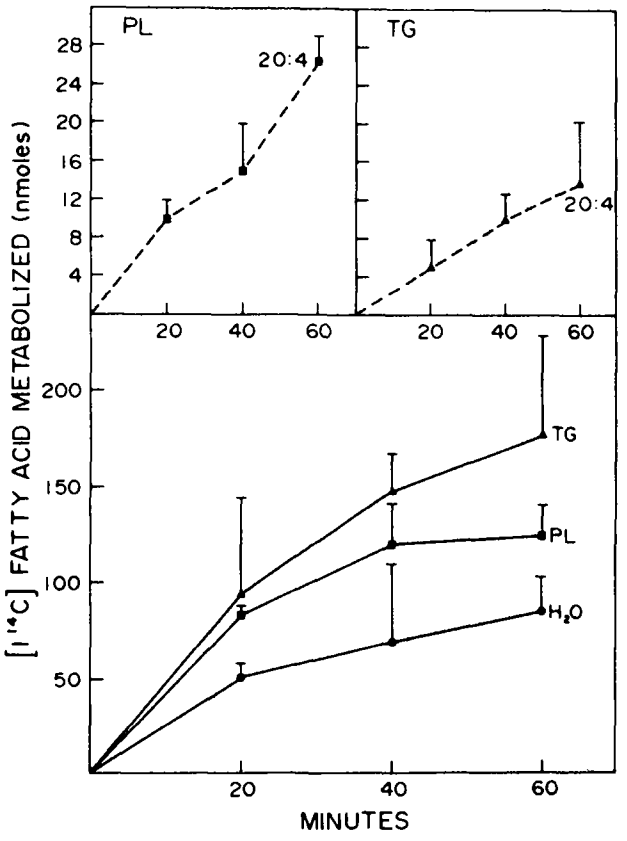

FIG. 6. Time-dependent metabolism of 0.3 mM [1-^{14}C]8,11,14-eicosatrienoic acid (2 Ci/mol). The conditions are as described in Figure 2. Results are the mean ± the standard deviation of four experiments.

TABLE 1

Metabolism of 0.3 mM [1-^{14}C]Linoleic Acid in the Presence of 6,9,12-Octadecatrienoic Acid or 8,11,14-Eicosatrienoic Acid

		mM Competition substrate					
		6,9,12-18:3[a]			8,11,14-20:3[a]		
Fatty acid	0[b]	0.15	0.30	0.45	0.15	0.30	0.45
TG 18:2	148 ± 14	137 ± 28	170 ± 13	184 ± 20	146 ± 1	146 ± 8	137 ± 23
TG 20:2	2.0 ± 0.7	0	0	0	0.3 ± 1	0	0
TG 18:3	1.3 ± 0.6	1.3 ± 1.3	1.8 ± 0.8	2.5 ± 0.4	1.4 ± 0.4	1.3 ± 0.4	1.6 ± 0.4
TG 20:3	1.9 ± 0.6	1.5 ± 0.1	0.6 ± 0.3	0.9 ± 0.1	1.5 ± 0.4	1.1 ± 0.1	0.9 ± 0.1
TG 20:4	0.9 ± 0.3	0.5 ± 0.4	0.4 ± 0.2	0	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
PL 18:2	72 ± 13	81 ± 8	82 ± 1	77 ± 1	93 ± 19	85 ± 7	75 ± 2
PL 20:2	3.1 ± 0.7	0.2 ± 0.2	0.2 ± 0.1	0	0.7 ± 0	0.3 ± 0	0
PL 18:3	1.1 ± 1.1	0.8 ± 0.3	1.3 ± 0.1	0.9 ± 0.4	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.5
PL 20:3	1.0 ± 0.6	1.2 ± 0.3	0.9 ± 0.1	0.5 ± 0	1.1 ± 0.3	0.7 ± 0.2	0.8 ± 0.1
PL 20:4	2.5 ± 1.4	1.1 ± 0.7	0.4 ± 0	0.5 ± 0	0.4 ± 0.1	0.3 ± 0	0.2 ± 0.1

Results are shown as nmol of (n-6) acids in triglyceride (TG) or phospholipids (PL) after a 40-min incubation.

[a]Values are average ± the range of two separate experiments.

[b]Values are means ± the standard deviations of six experiments.

that these cells have an enormous capacity to incorporate exogenous acids into lipids. If the small amount of radioactive 6,9,12-18:3 made from [1-^{14}C]linoleate totally mixes with exogenous 6,9,12-18:3, the specific activity of the total intracellular pool of 6,9,12-18:3 should be very low. Under these conditions, it would be virtually impossible to detect the synthesis of radioactive arachidonate. The results in Table 1 show that exogenous 6,9,12-18:3 almost totally blocked the chain elongation of [1-^{14}C]-linoleate to radioactive 11,14-20:2. These results are consistent with studies showing that a common malonyl-CoA condensing enzyme may act on unsaturated primers (13,14). In addition, 6,9,12-18:3 is a better substrate for chain elongation than is 9,12-18:2 (15). Exogenous 6,9,12-18:3 did not markedly depress the synthesis of radioactive 6,9,12-18:3 from [1-^{14}C]linoleate. Conversely, exogenous 6,9,12-18:3 inhibited, but did not totally block, the synthesis of radioactive 8,11,14-20:3 and arachidonate. These findings suggest that some linoleate may be channeled directly to arachidonate without total intracellular mixing of 6,9,12-18:3 and 8,11,14-20:3.

This conclusion is substantiated by the crossover inhibition studies shown in Table 2. These results compare how [1-^{14}C]6,9,12-18:3 was metabolized when incubations were carried out in the presence of increasing amounts of linoleate. Although there was considerable variation between different preparations of hepatocytes, these results show that concentrations of exogenous linoleate below 0.45 mM did not markedly influence [1-^{14}C]-6,9,12-18:3 metabolism. It is equally important to note that there was unreacted [1-^{14}C]6,9,12-18:3 in these incubations. When incubations contained 0, 0.15, 0.30 and 0.45 mM 18:2(n-6), there were 32, 79, 103 and 104 nmol of unreacted [1-^{14}C]6,9,12-18:3. Because these are direct crossover studies of those described in Table 1, the results document that when cells were incubated with [1-^{14}C]-linoleic acid and 6,9,12-18:3, some of the latter substrate still was available to compete with the [1-^{14}C]6,9,12-18:3 that was produced by desaturation of [1-^{14}C]9,12-18:2.

The results in Table 3 show that linoleate did not depress the conversion of radioactive [1-^{14}C]8,11,14-20:3 to arachidonate. The crossover inhibitor studies in Table 1 show that small amounts of linoleate were metabolized to arachidonate in the presence of 8,11,14-20:3. Presumably, small amounts of linoleate also are metabolized to arachidonate in the studies described in Table 3. However, the low levels of 8,11,14-20:3 made from linoleate would not significantly alter the intracellular pool of 8,11,14-20:3 because there still were 238 nmol of unreacted [1-^{14}C]-8,11,14-20:3 remaining when the incubations contained 0.45 mM linoleate.

DISCUSSION

The studies of Christophersen et al. (5), as well as those reported here, show that when hepatocytes are incubated

TABLE 2

Metabolism of 0.3 mM [1-^{14}C]6,9,12-Octadecatrienic Acid in the Presence of Linoleic Acid

	mM Linoleic acid			
Fatty acid	0	0.15	0.30	0.45
TG 18:3	99 ± 27	109 ± 43	107 ± 8	158 ± 30
TG 20:3	4 ± 2.4	1.9 ± 2.4	3.0 ± 3.1	1.9 ± 0.5
TG 20:4	1.4 ± 1.0	1.1 ± 1.1	1.2 ± 1.2	1.1 ± 0.5
PL 18:3	62 ± 15	67 ± 1	64 ± 7	53 ± 5
PL 20:3	3.0 ± 2.7	2.7 ± 3	2.9 ± 3.2	1.7 ± 0.1
PL 20:4	5.1 ± 1.9	4.2 ± 4.2	4.2 ± 3.8	2.1 ± 1.3

Results are shown as nmol of (n-6) acids in triglycerides (TG) or phospholipid (PL) after a 40-min incubation. Values are means ± the standard deviation of at least four experiments. The values for incubations containing 0.15 mM linoleic acid are the mean ± the range of two experiments.

TABLE 3

Metabolism of 0.3 mM[1-^{14}C]8,11,14-Eicosatrienoic Acid in the Presence of Linoleic Acid

	mM Linoleic acid			
Fatty acid	0	0.15	0.30	0.45
TG 20:3	124 ± 25	122 ± 46	127 ± 56	136 ± 55
TG 20:4	10.4 ± 3	12 ± 4	12 ± 4	14 ± 4
PL 20:3	81 ± 24	65 ± 12	68 ± 10	57 ± 6
PL 20:4	18.7 ± 5.5	14.7 ± 1.5	18.3 ± 2.3	17.2 ± 1.6

Results are shown as nmol of (n-6) acids in triglycerides (TG) or phospholipids (PL) after a 40-min incubation. Values are means ± the range of two separate experiments.

with [1-^{14}C]linoleate, their lipids contain radioactive 6,9,12-18:3, 8,11,14-20:3, and arachidonate. Conversely, compositional analysis of liver phospholipids usually fail to detect any 6,9,12-18:3 and only low levels of 8,11,14-20:3. Because desaturation of linoleate to 6,9,12-18:3 is rate-limiting in the production of arachidonate, the intracellular concentration of 6,9,12-18:3 must be very low. The intracellular fate of 6,9,12-18:3 then must be defined by K_m-values for the enzymes that incorporate it into phospholipids vs that for the malonyl-CoA condensing enzyme, which in turn is the rate-limiting step for microsomal chain elongation of fatty acids (13). According to this hypothesis, the K_m for the malonyl-CoA-dependent condensing enzyme should be lower than for the enzymes that acylate 6,9,12-18:3 into lipids. However, this hypothesis is not consistent with hepatocyte studies that show 6,9,12-18:3 is, in part, incorporated into lipids when these cells are incubated with linoleate. We reported that when hepatocytes were incubated with [1-^{14}C]-6,9,12-18:3 or [1-^{14}C]6,9,12,15-18:4, both of these substrates were initially rapidly incorporated into lipids (12). Over time, the amounts of 6,9,12-18:3 and 6,9,12,15-18:4 in lipids declined, but this decline was accompanied by an increase in long-chain radioactive (n-6) and (n-3) acids. These findings are at least partially consistent with rate studies. Lands et al. (6) reported that the rate of acylation of 6,9,12-18:3CoA by 1-acyl-GPC-transferase was 28 nmol/min/mg/rat liver microsomal protein. Bernert and Sprecher (4) reported that 4.4 nmol of 6,9,12-18:3 were chain-elongated to 8,11,14-20:3 per min/mg of rat liver microsomal protein. Collectively, these rate studies show that substantial amounts of 6,9,12-18:3 should be incorporated into phospholipids. However, the observed rapid release of 6,9,12-18:3 from hepatocyte lipids suggests that it enters a very small labile phospholipid pool and that it is rapidly released for subsequent metabolism to arachidonate (12).

The above results may, in part, explain why 6,9,12-18:3 is not detected when the fatty acid composition of liver lipids is measured. However, these findings alone probably cannot totally explain the type of labeling pattern that was observed when hepatocytes were incubated with [1-^{14}C]linoleate. Even at relatively short incubation times, it was possible to detect radioactive arachidonate in both triglycerides and phospholipids. This observation suggests that some linoleate may be channeled directly to arachidonate without total intracellular mixing of 6,9,12-18:3 or 8,11,14-20:3. This conclusion is supported by the finding that exogenous 6,9,12-18:3 inhibited but did not totally block the conversion of radioactive linoleate to arachidonate. Collectively, these studies as well as other hepatocyte studies (5,7,8,12) clearly show that measuring individual reaction rates for either phospholipid or fatty acid biosynthesis cannot in themselves be used to explain the types of polyunsaturated fatty acids found in liver lipids.

ACKNOWLEDGMENTS

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REFERENCES

1. Sprecher, H., and James, A.T. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.) pp. 303–338, American Oil Chemists' Society, Champaign, IL.
2. Paulsrud, J.R., Stewart, S.E., and Holman, R.T. (1970) *Lipids 5*, 611–616.
3. Brenner, R. (1974) *Mol. Cell Biochem. 3*, 41–52.
4. Bernert, J.T., and Sprecher, H. (1975) *Biochim. Biophys. Acta 398*, 354–363.
5. Christophersen, B.O., Hagve, T-A., and Norseth, J. (1982) *Biochim. Biophys. Acta 712*, 305–314.
6. Lands, W.E.M., Inoue, M., Suglura, V., and Okuyama, H.C. (1982) *J. Biol. Chem. 257*, 14968–14972.
7. Hagve, T-A., and Christophersen, B.O. (1983) *Biochim. Biophys. Acta 753*, 339–349.
8. Hagve, T-A., and Christophersen, B.O. (1984) *Biochim. Biophys. Acta 796*, 205–217.
9. Sprecher, H., and Lee, C.J. (1975) *Biochim. Biophys. Acta 388*, 113–125.
10. Sprecher, H., and Sankarappa, S.K. (1982) *Methods Enzymol. 86*, 357–366.
11. Seglen, P.C. (1976) *Methods Cell Biol. 13*, 29–83.
12. Voss, A.C., and Sprecher, H. (1988) *Biochim. Biophys. Acta 958*, 153–162.
13. Bernert, J.T., and Sprecher, H. (1977) *J. Biol. Chem. 252*, 6736–6744.
14. Nagai, M.N., Cook, L., Prasad, R., and Cinti, D. (1986) *Biochem. Biophys. Res. Commun. 140*, 74–80.
15. Ludwig, S. and Sprecher, H. (1979) *Arch. Biochem. Biophys. 197*, 333–341.

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Effects of Dietary Corn Oil and Salmon Oil on Lipids and Prostaglandin E_2 in Rat Gastric Mucosa

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Three groups of male rats were fed either a corn oil-enriched diet (17%, w/w), a salmon oil-enriched diet (12.5%) supplemented with corn oil (4.5%) or a low-fat diet (4.4%) for eight wk to investigate the possible relationships between dietary fatty acids and lipid composition, and prostaglandin E_2 level and phospholipase A_2 activity in the rat gastric mucosa.

High-fat diets induced no important variation in total protein, phospholipid and cholesterol contents of gastric mucosa.

Compared with a low-fat diet, corn oil produced a higher n-6/n-3 ratio in mucosal lipids, whereas this ratio was markedly lowered by a fish oil diet.

In comparison with the low-fat diet, the production of prostaglandin E_2(PGE_2) in gastric mucosa of rats fed salmon oil was significantly decreased by a factor of 2.8. In the corn oil group, PGE_2 production tended to decrease, but not significantly.

In comparison with the low-fat diet, both specific and total gastric mucosal phospholipase A_2 activities were increased (+ 18 and 23%, respectively) in the salmon oil group; they were unchanged in the corn oil group.

It is suggested that the decrease of gastric PGE_2 in rats fed fish oil is not provoked by a decrease in phospholipase A_2 activity but may be the result of the substitution of arachidonic acid by n-3 PUFA or activation of PGE_2 catabolism.

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It now is well accepted that prostaglandins can protect the gastric mucosa from becoming injured by various irritants and that this effect is mediated by two distinct mechanisms: inhibition of gastric acid secretion (1,2) and cytoprotection (3,4). It recently has become evident that exogenous and endogenous prostaglandins, mostly of the E-series (PGE_2), exert cytoprotective properties (3) and are effective in the treatment of peptic ulcer disease (5).

Synthesis of prostaglandins is initiated by the release of arachidonic acid through the action of phospholipase A_2 on the 2-position of membrane phospholipids. Such an activity has been described in rat gastric mucosa (6–8), and we recently have shown that this enzyme is mostly associated with microsomal and plasma membranes (8).

Prostaglandin synthesis can be affected by ingestion of fish oils. Fish oils are rich in n-3 polyunsaturated fatty acid (PUFA), which were demonstrated to be efficient in lowering plasma cholesterol and preventing atherosclerosis (9–14). Another beneficial effect of fish oils is to lower platelet aggregability through modifications of prostaglandin I_2 and thromboxane A_2 production (9,15). However, modification of the prostaglandin level in various tissues may lead to nonbeneficial effects. For example, Lokesh et al. (16) have recently reported that menhaden oil fed to mice altered the spleen prostaglandin production, which in turn may affect the immune function of this organ. In the same way, ingestion of cod liver oil by rats resulted in a reduction in kidney PGE_2 generation, which may influence blood pressure regulation (17).

In regard to the importance of prostaglandins in gastric cytoprotection and to the marked effect of the dietary n-3 PUFA on prostaglandin level in various tissues, we investigated the effect of n-3 PUFA (salmon oil) in comparison with n-6 PUFA (corn oil) on gastric prostaglandin production in rat. Possible relationships between dietary fatty acids and lipid composition of gastric mucosa, gastric phospholipase A_2 activity and prostaglandin production were studied.

MATERIAL AND METHODS

Animals and feeding procedures. Three groups of male Wistar rats (IFFA-Credo, L'Arbresle, France) weighing 190–210 g were used. Each group was divided into cages of two rats. One group (10 rats) was fed a low-fat diet containing (4.4%, w/w) of fat consisting of a lard and corn oil mixture giving a P/S ratio of 1.2. A second group (10 rats) received a corn oil-enriched diet (17%, w/w) with a P/S ratio of 5. The third group was fed a salmon oil-enriched diet (12.5%, w/w) supplemented with 4.5% of corn oil, with a P/S ratio of 2.0. The composition of the diets and their fatty acid compositions are given in Tables 1 and 2, respectively.

Corn oil, as supplied, contained 45 mg/100 g of α-tocopherol and salmon oil was supplemented with 100 mg/100 g of α-tocopherol as antioxidant. So, the total amounts supplied by the control diet, corn oil diet and salmon oil diet were 171 mg, 246 mg and 315 mg/kg of diet, respectively. The low-fat and corn oil diets were prepared to last one month and stored at −20 C in plastic bags, and the salmon oil diet was prepared every two wk and stored at −20 C in sealed containers flushed with nitrogen.

The rats were fed ad libitum, and uneaten food was discarded in the morning. They had free access to water, and the feeding period was for eight wk.

Procedures for stomach. The animals were fasted overnight, weighed and guillotined. Their stomachs were removed, opened and extensively washed at 4 C with the homogenization buffer (5 mM Tris HCl, pH 7.4, 0.25 M sucrose). The upper portion of the stomach, the forestomach, was discarded, and the glandular part (antrum plus fundus) was gently scraped on an ice-cold glass plate, the mucosa was weighed and suspended in the same homogenization buffer (10%, w/v) and homogenized by

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Abbreviations: FAME, fatty acid methyl esters; PA_2, phospholipase A_2; PGE_2, prostaglandin E_2; PGI_2, Prostacycline I_2; PGI_3, Prostacycline I_3; PUFA, polyunsaturated fatty acids; ANOVA, analysis of variance; GLC, gas liquid chromatography; P/S ratio, polyunsaturated to saturated fatty acid ratio; RIA, radioimmunoassay.

TABLE 1

Composition of Experimental Diets

	Low fat g/100 g	Corn oil g/100 g	Salmon oil g/100 g
Casein[a]	27.3	27.3	27.3
Lard[a]	2.2	—	—
Salmon oil[b]	—	—	12.5
Corn oil[c]	2.2	17.0	4.5
Starch[a]	34.0	26.9	26.9
Glucose[a]	23.5	18.0	18.0
Minerals[a]	5.0	5.0	5.0
Vitamins[a]	1.0	1.0	1.0
Cellulose[a]	4.8	4.8	4.8
BHT[d]	0.05	0.05	0.05

[a]From Unité Alimentation Rationnelle (UAR) (Villemoisson, France).

[b]From SEAH International (Boulogne/Mer France).

[c]From CPC Europe Consumer Products (Heilbronn, FRG).

[d]Butylated Hydroxytoluene, Sigma, Coger (Paris, France).

Vitamins, given in g/kg diet: retinol, 19.8 IU; cholecalciferol, 6 IU; thiamin, 0.02; riboflavin, 0.015; nicotinamide, 0.035; pyridoxine, 0.01; carnitine, 0.15; cyanocobalamin, 5×10^{-5}; ascorbic acid, 0.8; α-tocopherol, 0.17; menadione, 0.04; nicotinic acid, 0.10; choline, 1.36; pteroylmonoglutamic acid, 5×10^{-4}; p-aminobenzoic acid, 0.05; biotin, 3×10^{-4}. Minerals given in g/kg diet: $CaHPO_4$ 21.5; KCl, 5; NaCl, 5; $MgSO_4$, 2.50; Fe_2O_3, 0.15; $FeSO_4 \cdot 7H_2O$, 0.25; $MnSO_4 \cdot H_2O$, 0.12; $CuSO_4 \cdot 5H_2O$, 0.025; $CoSO_4 \cdot 7H_2O$, 2×10^{-4}; $ZnSO_4 \cdot 7H_2O$, 0.1; stabilized KI, 4×10^{-4}; NaF, 0.012. UAR, Villemoisson/Orge, France. On the basis of UAR information, the selenium content of the diets was estimated at 50–60 μg/kg.

TABLE 2

Fatty Acid Composition of the Diets (%)

Fatty acid	Low fat	Corn oil	Salmon oil
14:0	—	—	4.6
15:0	—	—	0.3
16:0	20.2	11.6	12.6
16:1n-7	0.3	—	5.4
17:0	—	—	0.6
18:0	8.0	0.2	1.8
18:1n-9	35.1	26.8	14.8
18:1n-7	—	—	2.6
18:2n-6	33.6	60.0	16.9
18:3n-3	0.7	0.8	0.6
18:4n-3	—	—	2.0
20:0	0.3	0.4	0.3
20:1n-9	0.5	—	2.6
20:1n-11	—	—	7.8
20:4n-6	0.3	0.1	0.4
20:4n-3	—	—	0.6
20:5n-3	—	—	9.8
22:1n-11	—	—	6.6
22:1n-9	—	—	0.6
22:5n-3	—	—	1.2
22:6n-3	—	—	7.2
24:1n-9	—	—	0.4
Saturated	28.6	12.2	20.0
Polyunsaturated	34.5	61.3	39.8
P/S	1.2	5.0	2.0
n-6/n-3	46.0	75.0	0.8

using a polytron tissue processor (Kinematica PT-10, Luzern, Switzerland) for 10 sec at the rheostat setting of 6.0.

Fractions for the prostaglandin E_2 assay were diluted with a same volume (v/v) of a Tris HCl 0.1 M, pH 7.4, buffer to obtain a final concentration of 50 mM Tris HCl, pH 7.4, 0.125 M sucrose. Then they were stored at −60 C until use, and undiluted fractions were stored at −20 C pending biochemical analysis.

Protein assay. Proteins were measured by the dye-binding method of Bradford (18) using rabbit γ-globulin as standard (protein assay kit, Bio-Rad, Richmond, CA).

Phospholipase A_2 assay. Gastric phospholipase A_2 was assayed as recently described (8). Incubation mixtures contained 0.2 mM 1-palmitoyl-2[1-^{14}C]oleoylphosphatidylcholine (Amersham, France) with a specific radioactivity of 59.2 KBq/μmol and 50 mM sodium cacodylate at pH 7.0 in a final volume of 0.2 ml. Radioactivity was counted by liquid scintillation spectrometry (Beckman LS 9000, Palo Alto, CA) in 10 ml of Readysolve MP (Beckman) scintillation fluid. The results were corrected for a control incubated without protein. All the assays were done in duplicate.

Prostaglandin E_2 assay. Prostaglandin E_2 was extracted with ethylacetate from gastric mucosa homogenates (5 mg protein) according to the method of Lokesh et al. (16). Prostaglandin E_2 was quantified by radioimmunoassay (RIA), using a [^{125}I]prostaglandin E_2 RIA kit (NEK-020) purchased from NEN (Paris, France).

Appropriate blanks for nonspecific binding and tubes for total binding determinations were included. All the assays were done in duplicate (two extractions). All the measurements were in the linear portion of the standard curve.

Lipid analysis. Lipids were extracted from the gastric mucosa homogenate by the method of Folch et al. (19).

A fraction of this extract was used for lipidic phosphorus determination (20). Another part of this extract was used to determine total lipid fatty acid composition. Fatty acid methyl esters (FAME) of total lipids were prepared according to a rapid and convenient method used for vegetable oils (21). FAME recovered in hexane were stored under nitrogen at −20 C pending gas liquid chromatography (GLC) analysis. Separation of FAME was performed as described (22), using a gas liquid chromatograph (Girdel 3000, Paris, France) equipped with a peak integrator (Delsi, Enica 10, Suresne, France) and a 50 m capillary column (Spirawax FS, 1493, Spiral, Dijon, France).

The cholesterol content of gastric mucosa was determined with the cholesterol esterase-cholesterol oxidase kit (Boehringer Mannheim, Mannheim, FRG) using a two-point kinetic method. Measurements were made with an automatic Multistat III (Instrumentation Laboratory, Lexington, MA) at 30 C.

Statistical analysis. Results presented in the tables are mean ±SD. Statistical significance of mean differences between dietary groups was investigated by analysis of variance (ANOVA) and by the multiple comparison of Scheffé at $p < 0.05$ or $p < 0.01$.

RESULTS

Over the eight-wk feeding period, there was no significant difference ($p > 0.05$) in body weight gains. They were (in

g): 180.5 ± 19.3, 203.1 ± 35.0 and 218.0 ± 25.0 for rats fed low-fat, corn oil and salmon oil diets, respectively. Also, gastric mucosa weights were not significantly different among the three groups of rats: they weighed (in g) 0.37 ± 0.03, 0.33 ± 0.06 and 0.37 ± 0.06 in rats fed low fat, corn oil and salmon oil, respectively. No visible gastric ulceration was observed among the three groups of rats.

The content of proteins, total phospholipids and total cholesterol of gastric mucosa is shown in Table 3. In comparison with a low-fat diet, only corn oil induced a moderate increase of 15% in proteins and a slight decrease of 14% in total phospholipids. Total cholesterol was unchanged among the three dietary groups.

To estimate the respective contribution of fatty acid originated from phospholipids and triglycerides, we gravimetrically determined the total amount of gastric mucosa lipids obtained from the Folch extraction. The quantification of lipidic phosphorus and cholesterol in this extract allowed us to estimate the amount of neutral lipids. It showed that the phospholipids represented about 75% (w/w) of the lipids esterified by fatty acids.

The fatty acid composition of total lipids is shown on Table 4. It can be observed that in comparison with the low fat diet, both corn oil and salmo • oil induced profound modifications in the fatty acid composition of gastric lipids. Corn oil diet provoked a significant decrease in both saturated (−40%) and monounsaturated (−20%) fatty acids, whereas PUFA increased two-fold. This rise was due mostly to a three-fold increase in linoleic acid. However, arachidonic acid was significantly decreased by a factor of 1.4. All of these modifications led to a 1.7-fold increase in the n-6/n-3 ratio. Salmon oil–enriched diet provoked a less-pronounced decrease in saturated and monounsaturated fatty acids than corn oil did. In comparison with a low-fat diet, PUFA were 45% higher. In the PUFA, the most pronounced effect of salmon oil was a 2.4-fold decrease in arachidonic acid, whereas n-3 PUFA were drastically increased, particularly the 20:5n-3 and 22:6n-3. Consequently, in comparison with the two other dietary groups, a drastic reduction in the n-6/n-3 ratio and in the 20:4n-6/20:5n-3 ratio was obtained in this dietary group, whereas the unsaturation index was augmented by about 38%.

The activity of gastric phospholipase A_2 and the PGE_2 production under the various dietary conditions were investigated. Results presented in Table 5 show that in comparison with the low-fat diet, only the salmon oil–enriched diet induced a slightly significant increase (+18%) in the specific activity of phospholipase A_2. In the same way, the total phospholipase A_2 activity (μmol/hr/g mucosa) also was slightly increased (+23%) in the salmon oil group. In this last group, compared with the low-fat group, PGE_2 production was considerably lowered by a factor of 2.8. Although the PGE_2 level tended to decrease in the corn oil group, the difference was not significant ($p > 0.05$) compared with the low-fat diet group. However, this PGE_2 level still was significantly ($p < 0.01$) higher (2.1 times) than that of the salmon oil group.

DISCUSSION

Throughout the feeding period, all the diets were well-accepted by the rats as judged by their growth rates. The diets induced no visible ulceration of the gastric mucosa. Protein and lipid composition of mucosa showed no important variation under the different dietary conditions.

Significant modifications were seen in the fatty acid composition of total lipids. Generally speaking, the modifications observed seem to reflect the fatty acid composition of the diet. For the corn oil diet, the decrease in saturated fatty acids was associated with a high increase in linoleic acid. This may be the result of a high affinity of acyl-CoA:lysophosphatidylcholine-acyltransferase for the linoleoyl-CoA during reconstitution of membrane phospholipids initially hydrolyzed by the active gastric phospholipase A_2 (8). Curiously, this increase in linoleic acid was not correlated with a higher conversion into arachidonic acid, the final level of which even decreased. This phenomenon also has been observed in various tissues like kidney (17), heart (22) and platelets (23,24) of animals fed linoleic-rich vegetable oils. This could indicate that, at some point, a balance is reached between the increased availability of linoleic acid and its conversion into arachidonic acid. The results obtained with salmon oil–enriched diet show that n-3 PUFA of the diet are easily incorporated in gastric mucosa lipids, drastically reducing

TABLE 3

Effects of Dietary Corn Oil and Salmon Oil on Protein, Lipidic Phosphorus and Total Cholesterol Content of Rat Gastric Mucosa

	Low fat	Corn oil	Salmon oil
Protein mg protein/g mucosa	195.7 ± 22.1^{a,*}	225.9 ± 15.2^{b,*}	204.4 ± 21.2^{a,b}
Lipidic phosphorus μmol/g mucosa	8.0 ± 1.8^{a,b}	6.9 ± 0.9^{a,**}	9.2 ± 1.2^{b,**}
Total cholesterol μmol/g mucosa	4.8 ± 1.2	4.9 ± 1.0	4.1 ± 1.1

Values without a common superscript are statistically different at *$p < 0.05$; **$p < 0.01$. If no superscript appears, values are not different ($p > 0.05$). Values are mean ± SD, and n = 10 for low-fat and corn oil diets, n = 12 for salmon oil diet.

TABLE 4

Effects of Dietary Corn Oil and Salmon Oil on Total Lipid Fatty Acid Composition of Rat Gastric Mucosa

Fatty acid %	Low fat	Corn oil	Salmon oil
14:0	1.43 ± 0.03^{a,**}	0.93 ± 0.07^{b,**}	3.15 ± 0.31^{c,**}
16:0	26.42 ± 0.65^{a,**}	15.25 ± 0.56^{b,**}	20.50 ± 1.67^{c,**}
16:1n-9	tr	0.85 ± 0.07	1.00 ± 0.22
16:1n-7	4.67 ± 0.74^{a,**}	1.10 ± 0.39^{b,**}	3.85 ± 0.35^{a,**}
18:0	10.06 ± 1.26^{a,**}	6.21 ± 0.85^{b,**}	9.50 ± 1.27^{a,**}
18:1n-9	29.34 ± 2.89^{a,**}	25.40 ± 0.66^{b,**}	21.20 ± 1.47^{c,**}
18:1n-7	4.27 ± 0.48^{a,*}	2.10 ± 0.15^{b,*}	3.90 ± 0.30^{a,*}
18:2n-6	10.33 ± 1.51^{a,**}	35.60 ± 0.97^{b,**}	13.90 ± 1.01^{c,**}
18:3n-3	0.10 ± 0.01	0.52 ± 0.01	0.50 ± 0.09
20:0	1.06 ± 0.70	0.93 ± 0.11	1.60 ± 0.37
20:4n-6[1]	9.53 ± 2.30^{a,*}	6.90 ± 1.23^{b,*}	4.00 ± 0.62^{c,*}
20:5n-3	0.16 ± 0.09^{a,**}	tr	5.00 ± 0.44^{b,**}
22:1n-11	tr	1.10 ± 0.15^{a,**}	2.50 ± 0.35^{b,**}
22:4n-6	1.69 ± 1.33	1.90 ± 0.27	1.50 ± 0.50
22:5n-3	tr	tr	1.70 ± 0.11
22:6n-3	1.02 ± 0.63^{a,**}	1.00 ± 0.19^{a,**}	6.40 ± 0.21^{b,**}
Saturated	38.97 ± 1.49	23.92 ± 1.49	34.75 ± 2.35
Monounsaturated	38.28 ± 3.60	30.55 ± 0.82	32.25 ± 2.22
Polyunsaturated	22.83 ± 0.97	45.92 ± 1.25	33.00 ± 1.48
n-6	21.55 ± 0.99	44.40 ± 1.12	19.40 ± 0.99
n-3	1.28 ± 0.57	1.52 ± 0.18	13.60 ± 0.73
n-6/n-3 ratio	16.83	29.21	1.42
$\frac{20:4n-6}{20:5n-3}$ ratio	59.56	∞	0.80
UI	111.70	114.51	155.45

Values without a common superscript are statistically different at $^{*}p < 0.05$; $^{**}p < 0.01$. If no superscript appears, values are not different ($p > 0.05$). Values are mean ± SD and n = 5 for low-fat and corn oil diet, n = 6 for salmon oil diet, and each n represents the pool of two gastric mucosa.

[1]For this fatty acid, salmon oil value and low-fat value are statistically different at $p < 0.01$.

tr, Traces < 0.1%.

UI, unsaturation index: addition of (percent of each fatty acid × double bond/fatty acid).

TABLE 5

Effects of Dietary Corn Oil and Salmon Oil on Phospholipase A_2 Activity and PGE_2 Production of Rat Gastric Mucosa

	Low fat	Corn oil	Salmon oil
Phospholipase A_2 Specific activity nmol/hr/mg protein	628.4 ± 44.0^{a,**}	568.5 ± 57.4^{a,**}	741.7 ± 35.0^{b,**}
Phospholipase A_2 Total activity μmol/hr/g mucosa	123.3 ± 18.8^{a,**}	126.5 ± 17.6^{a,*}	151.7 ± 19.4^{b,**}
PGE_2 ng/g mucosa	327.3 ± 75.4^{a,**}	250.8 ± 82.4^{a,**}	116.5 ± 40.9^{b,**}

Values without a common superscript are statistically different at $^{*}p < 0.05$; $^{**}p < 0.01$. If no superscript appears, values are not different ($p > 0.05$). Values are mean ± SD. For phospholipase A_2, n = 10 for low-fat and corn oil diets; n = 12 for salmon oil diet. For PGE_2 values: n = 9 for low-fat diet; n = 8 for corn oil diet; n = 12 for salmon oil diet.

both the n-6/n-3 and the 20:4n-6/20:5n-3 ratios. The marked reduction of arachidonic acid could not be attributed to the lack of the 18:2n-6 substrate in the precursor pool because an adequate amount of this fatty acid was added to the diet by supplementing with corn oil. Such a competitive incorporation of dietary n-3 PUFA also has been observed in various tissue phospholipids in rat (15–17,25,26). This enhanced incorporation of n-3 PUFA may be the result of several factors, including a better esterification of n-3 PUFA into membrane phospholipids by acyltransferase, or a competition between n-3 PUFA and 18:2n-6 at desaturation step (27) or a direct inhibition of the Δ6-desaturase by the 22:6n-3 (28).

Arachidonic acid is the precursor of the prostaglandins of the 2-series, whereas eicosapentaenoic acid is the precursor of the 3-series. The salmon oil–enriched diet sharply decreased the ratio 20:4n-6/20:5n-3 in gastric phospholipids, which may have important consequences on the gastric prostaglandin level. Among the gastric mucosal prostaglandins, PGE_2 was demonstrated to be more efficient in protecting (3,29) and in treating peptic ulcer diseases (5), therefore we focused our study on this prostaglandin. Clearly, fish oil induced a significant and sharp decrease in gastric PGE_2 production. Reduction of PGE_2 level also was observed in spleen (16) and kidney (17) of animals fed fish oils. The PGE_2 decrease is certainly not the result of a reduction in the release of the precursor 20:4n-6 from membrane phospholipids by the gastric phospholipase A_2, because its specific activity was slightly increased in this dietary group. This PGE_2 decrease can result from the drop in the arachidonic acid pool. Although not significant, this phenomenon also was observed in the corn oil group in which the drop in arachidonic acid might explain that PGE_2 tended to decrease. Reduction in PGE_2 levels also has been noted in other studies in which animals were fed linoleic acid-enriched oils (17,23,30) or in which endothelial cells were cultured with linoleic acid (31,32).

In addition to the diminution of the arachidonic pool, the presence in fish oil diet of n-3 PUFA may exert a direct inhibitory action on the cyclooxygenase (33,34). Combination of these effects may explain the drastic drop in gastric PGE_2 level production in rats fed salmon oil. Lastly, a PUFA-induced enhanced prostaglandin peroxisomal degradation also may lead to a lower PGE_2 level as judged by results in progress that show an increase in the activity of some peroxisomal enzymes in gastric mucosa of animals fed fish oil.

In conclusion, this study brings an additional and original demonstration of the inhibitory effect of high doses of n-3 PUFA on prostaglandin generation in organs. In the case of gastric mucosa, this diminution could impair the cytoprotective properties of PGE_2. This, in turn, may hamper the resistance of the stomach to various injuries like stress, lysolecithins or bile salts after a duodenal reflux.

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REFERENCES

1. Robert, A., Schultz, J.R., Nezamis, J.E., and Lancaster, C. (1976) *Gastroenterology 70,* 359–370.
2. Konturek, S.J., and Pawlik, W. (1986) in *Biological Protection with Prostaglandins* (Cohen, M.M., ed.) Vol. II, pp. 45–61, CRC Press, Inc., Boca Raton, FL.
3. Robert, A., Nezamis, J.E., Lancaster, C., and Hanchar, A.J. (1979) *Gastroenterology 77,* 433–443.
4. Robert, A. (1984) *Gastroenterol. Clin. Biol. 8,* 819–827.
5. Vantrappen, G., Janssens, J., Propiela, T., Kulig, G., Tytgat, G.N.J., Huibregtse, K., Lambert, R., Pauchard, J.P., and Robert, A. (1982) *Gastroenterology 83,* 357–363.
6. Wassef, M.K., Lin, Y.N., and Horowitz, M.I. (1978) *Biochim. Biophys. Acta 528,* 318–330.
7. Hirohara, J., Sugatani, J., Okumura, T., Sameshima, Y., and Saito, K. (1987) *Biochim. Biophys. Acta 919,* 231–238.
8. Grataroli, R., Charbonnier, M., Léonardi, J., Grimaud, J.C., Lafont, H., and Nalbone, G. (1987) *Arch. Biochem. Biophys. 258,* 77–84.
9. Goodnight, S.H. Jr., Harris, W.S., Connor, W.E., and Illingworth, D.R. (1982) *Arteriosclerosis 2,* 87–113.
10. Dyerberg, J., Bang, H.O., Stofferson, E., Moncada, S., and Vane, J.R. (1978) *Lancet 2,* 117–119.
11. Hauge, J.G., and Nicolaysen, R. (1959) *Acta Physiol. Scand. 45,* 26–30.
12. Harris, W.S., Connor, W.E., and McMurry, M.P. (1983) *Metabolism 32,* 179–184.
13. Kromhout, D., Bosshieter, E.B., and Coulander, C.L. (1985) *N. Eng. J. Med. 312,* 1205–1209.
14. Durand, G., Pascal, G., Legrand, P., and Gounelle de Pontanel, H. (1985) *Med. Nutr. 21,* 391–406.
15. Iritani, N., and Fujikawa, S. (1982) *J. Nutr. Sci. Vitaminol. 28,* 621–629.
16. Lokesh, B.R., Hsieh, H.L., and Kinsella, J.E. (1986) *Ann. Nutr. Metab. 30,* 357–364.
17. Croft, K.D., Beilin, L.J., Vandongen, R., and Mathews, E. (1984) *Biochim. Biophys. Acta 795,* 196–207.
18. Bradford, M.M. (1976) *Anal. Biochem. 72,* 248–254.
19. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497–509.
20. Amic, J., Lairon, D., and Hauton, J.C. (1972) *Clin. Chim. Acta 40,* 107–114.
21. Cecchi, G., Biasini, S., and Castano, J. (1985) *Rev. Fr. Corps Gras 32,* 163–164.
22. Léonardi, J., Termine, E., Morand, F., Lafont, R., Portugal, H., Lafont, H., and Nalbone, G. (1987) *Lipids 22,* 517–522.
23. Galli, C., Agradi, E., Petroni, A., and Tremoli, E. (1981) *Lipids 16,* 165–172.
24. McGregor, L., Morazain, R., and Renaud, S. (1980) *Lab. Invest. 43,* 438–442.
25. Swanson, J.E., and Kinsella, J.E. (1986) *J. Nutr. 116,* 514–523.
26. Gudbjarnason, S., and Oskarsdottir, G. (1977) *Biochim. Biophys. Acta 487,* 10–15.
27. Sprecher, H. (1981) *Progr. Lipid Res. 20,* 13–22.
28. Brenner, R.R. (1982) *Progr. Lipid Res. 20,* 41–47.
29. Konturek, S.J., Piastucki, I., Brzozowski, T., Radecki, T., Dembinska-Kiec, A., and Griglewski, R. (1981) *Gastroenterology 80,* 4–9.
30. de Deckere, E.A.M., Nugteren, D.H., and Ten Hoor, F. (1979) *Prostaglandins 17,* 947–955.
31. Spector, A.A., Kaduce, T.L., Hoak, J.C., and Fry, G.L. (1981) *J. Clin. Invest. 68,* 1003–1011.
32. Spector, A.A., Hoak, J.C., Fry, G.L., Denning, G.H., Stoll, L.L., and Smith, J.B. (1980) *J. Clin. Invest. 65,* 1003–1012.
33. Culp, B.R., Titus, B.G., and Lands, W.E.M. (1979) *Prostaglandins Med. 3,* 269–278.
34. Corey, E.J., Shih, C., and Cashman, J.R. (1983) *Proc. Natl. Acad. Sci. USA 80,* 3581–3584.

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Perfluorodecanoic Acid and Lipid Metabolism in the Rat

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Alterations in lipid metabolism were examined in adult male Sprague-Dawley rats seven days after a single intraperitoneal injection of perfluorodecanoic acid (PFDA; 20, 40 or 80 mg/kg). Because PFDA treatment caused a dose-related reduction in feed intake, the response of vehicle-treated rats pair-fed to those receiving PFDA was monitored to distinguish direct effects of the perfluorinated fatty acid from those secondary to hypophagia. Carcass content of lipid phosphorus and free cholesterol decreased in dose-dependent fashion in both PFDA-treated and pair-fed rats. Carcass triacylglycerols diminished in a similar manner, yet PFDA-treated rats at each dose had a higher concentration of neutral acylglycerols than their vehicle-treated, pair-fed counterparts. In vehicle-treated, pair-fed rats at the 80 mg/kg dose level, lipid phosphorus and free cholesterol as a proportion of carcass fat increased, whereas the share of the triacylglycerols declined. Because of the higher concentration of triacylglycerols in the carcass of rats treated with 80 mg/kg PFDA, enrichment of lipid phosphorus and free cholesterol in carcass fat was less than in their pair-fed partners. The amount of lipid phosphorus and free cholesterol per hepatocyte was similar in both PFDA-treated rats and their pair-fed partners. Liver triacylglycerols were markedly increased in PFDA-treated rats. A similar but less extensive augmentary effect of PFDA on hepatic esterified cholesterol was found. Concentration of triacylglycerols in plasma was not elevated in PFDA-treated rats, in spite of hepatic accumulation of esterified compounds. Also, the plasma level of free fatty acids and 3-hydroxybutyrate was similar in all treatment groups, including those receiving PFDA. Thus, the administration of PFDA appears to divert fatty acids from oxidation toward esterification in the liver.
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Derivatives of perfluorosulfonic and perfluorocarboxylic acids have been used in a number of industrial applications as lubricants, plasticizers, wetting agents and corrosion inhibitors (1). Aqueous film-forming foams, used as fire extinguishants, contain mixtures of hydrocarbon and fluorocarbon surfactants (derivatized fatty acids) due to their superior surface-active properties (2).

Perfluorodecanoic acid (PFDA), representative of these perfluorinated fatty acids, resulted in a progressive reduction in feed intake, body weight loss, along with an increase in liver mass and changes in hepatic lipid composition in the rat (3,4). These effects followed treatment with a single intraperitoneal dose of PFDA. Recently, PFDA-treated rats were found to either gain less or lose more weight (depending on the dose administered) than vehicle-treated rats with the same caloric intake (5). Despite their lower body weight, PFDA-treated rats had a greater carcass fat content than their pair-fed counterparts (5).

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Abbreviations: PFDA, perfluorodecanoic acid; ANOVA, analysis of variance; SAS, statistical analysis system.

Lipid metabolism in the PFDA-treated adult male Sprague-Dawley rat has been examined in this study. By quantifying and comparing values for carcass, liver and plasma lipids in PFDA-treated and pair-fed animals, direct effects of PFDA on lipid metabolism were distinguished from those secondary to hypophagia.

MATERIALS AND METHODS

Chemicals. PFDA (also nonadecafluorodecanoic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and determined to have a purity of 87.4% by gas chromatography (6). All enzymes and cofactors were obtained from Boehringer Mannheim Corp. (Indianapolis, IN), with the exception of cholesterol oxidase and o-dianisidine, which were purchased from Sigma Chemical Co. (St. Louis, MO). Coomassie blue G-250 was obtained from Pierce Chemical Co. (Rockford, IL).

Animals. Male Sprague-Dawley rats (275–300 g), obtained from Harlan Sprague-Dawley (Indianapolis, IN), were individually housed in suspended stainless-steel cages in a temperature-controlled room (ca. 21 C) with a 12-hr light/dark cycle (lighted, 0500-1700 hr). A schedule was maintained so that ground feed (Purina Rat Chow, #5012, Ralston Purina Co., St. Louis, MO) was available from 1700 to 0830 hr and tap water was available ad libitum. An acclimation period of at least one wk was allowed before the initiation of an experiment. Rats then were paired on the basis of similar body weight. PFDA-treated rats received a single intraperitoneal injection of PFDA with dosing at 20, 40 or 80 mg/kg. At 24 hr after the PFDA-treated rat was dosed, its partner with a similar weight was given an equivalent volume of vehicle (propylene glycol/water, 50:50, v/v; 1 ml/kg). This vehicle-treated animal then received the same amount of feed its PFDA-treated partner had consumed during the previous 15–16 hr feeding period (pair-feeding). An additional group of vehicle-treated rats, with unlimited access to ground chow during the feeding period (15–16 hr), was included. Body weight and feed intake were measured daily for seven days following injection of PFDA or vehicle.

Experimental protocols. Two series of protocols were conducted. In the first set, carcass lipid was determined in rats killed by cervical dislocation seven days post-treatment between 1600 and 1700 hr. The gastrointestinal tract was removed, emptied of all contents and returned to the body cavity. Carcasses were weighed, placed in plastic bags, and frozen at −20 C. Each frozen carcass was ground to a homogeneous mixture with a Wiley laboratory mill cooled with liquid nitrogen. The ground carcasses were stored in individually sealed bags (Ziploc) at −20 C for later body composition analysis (5).

Liver and plasma lipid analyses were performed on tissue from rats exsanguinated by decapitation seven days post-treatment between 1300 and 1600 hr in the second series of protocols. Trunk blood was collected in a 50 ml disposable beaker containing 100 μl of 15% (w/v) potassium-ethylenediamine tetraacetic acid. Plasma was separated and stored at −70 C. A portion of liver was

frozen by freeze-stop technique (7) at the temperature of liquid nitrogen, weighed, and ground to a fine powder in a pre-cooled mortar and pestle. The frozen liver powder was stored in cryogenic vials under liquid nitrogen until the time of assay. The remaining liver tissue was excised and weighed. Total liver mass represents the combined weight of these two portions.

Analytical methods. Carcass, liver and plasma lipids were extracted with chloroform/methanol by a micro-adaptation of the procedure described originally by Folch et al. (8). Liver and carcass tissue powders, as well as plasma samples, were homogenized by mechanical disruption (2 × 15 sec with a 30-sec pause) in chloroform/methanol (2:1, v/v) in the proportion of 19 volumes of solvent to 1 volume of sample using a Tissumizer homogenizer with a micro probe (Tekmar Co., Cincinnati, OH). The crude extract was washed with 50 mM NaCl. Aliquots of the washed lipid extract were taken to dryness and analyzed for neutral glycerols (9). For lipid phosphorus determinations (10), digestion of lipid in a dried crude extract was accomplished by gentle refluxing with 70% perchloric acid in the presence of pure carborundum chips (133-A, Hengar and Co., Philadelphia, PA) on a micro-Kjeldahl digestion rack (11). Dipalmitoyl-dl-α-phosphatidylcholine, dipalmitoyl-dl-α-phosphatidylethanolamine, and sodium tribasic phosphate yielded equivalent standard curves. Determination of carcass fat (ether-extractable at 50 C) has been described (5).

Total cholesterol determination followed chemical hydrolysis of the cholesteryl esters to free cholesterol (12). Cholesterol then was oxidized enzymatically to cholest-4-en-3-one and hydrogen peroxide with cholesterol oxidase. The hydrogen peroxide generated was reacted with horseradish peroxidase in the presence of o-dianisidine to form a stable chromophore (13). Determination of free cholesterol was performed with the enzymatic reaction for cholesterol in the absence of chemical hydrolysis. Esterified cholesterol then was calculated as the difference between the values for total and free cholesterol. Enzymatic determination of 3-hydroxybutyrate was made on neutralized, perchloric acid extracts of plasma (14) as described by Williamson and Mellanby (15). The concentration of free fatty acids in plasma was quantitated by a sensitive micro method (16).

For DNA and protein analysis, liver homogenates in water (10%, w/v) were prepared by mechanical disruption (2 × 15 sec; Tissumizer) with a 30-sec pause between bursts with cooling in an ice-water mixture. After extraction of interfering lipids (17), DNA content was estimated by a micro-adaptation of the procedure described by Richards (18) using calf thymus DNA as standard. Total hepatic protein was determined by the dye-binding method of Bradford (19) with crystalline bovine serum albumin as standard.

Statistical analysis. The main effects of treatment (PFDA vs pair-fed) and dosing (20, 40 or 80 mg/kg) were analyzed by two-way analysis of variance (ANOVA) through the use of unweighted cell means (20). Significance of difference between PFDA-treated rats and their pair-fed counterparts at a given dose was detected by pair-wise comparison (20). Effect of dosing between treatment groups, PFDA or pair-fed, including comparison with the vehicle-treated group of rats with unlimited access to feed, was tested by one-way ANOVA and significance of differences between doses was analyzed using Scheffé's multiple comparison method (20). Linear function among doses was evaluated by testing for trends using orthogonal coefficients if effect of dosing in the one-way ANOVA was significant (21). The computations were performed with a VAX-750 computer using SAS (22). In all cases, significance was set at $p < 0.05$.

RESULTS

Body weight and feed intake. Cumulative feed intake of rats during the seven days following PFDA treatment was diminished in a dose-related manner with a remarkable decrease occurring at the 80 mg/kg dose (Fig. 1). Body weight of both PFDA-treated rats and their vehicle-treated, pair-fed counterparts at seven days post-treatment also was reduced in a dose-dependent fashion. Even though the cumulative feed intake of PFDA-treated rats and pair-fed counterparts was comparable at each dose level, the mean body weight of those receiving PFDA was lower at seven days after treatment (Fig. 1). When the change in body weight (calculated as the difference between the initial and final body weight) was analyzed by two-way ANOVA, the difference between PFDA-treated rats and their respective vehicle-treated, pair-fed partners was significant by pair-wise comparison at the 40 and 80 mg/kg dose levels.

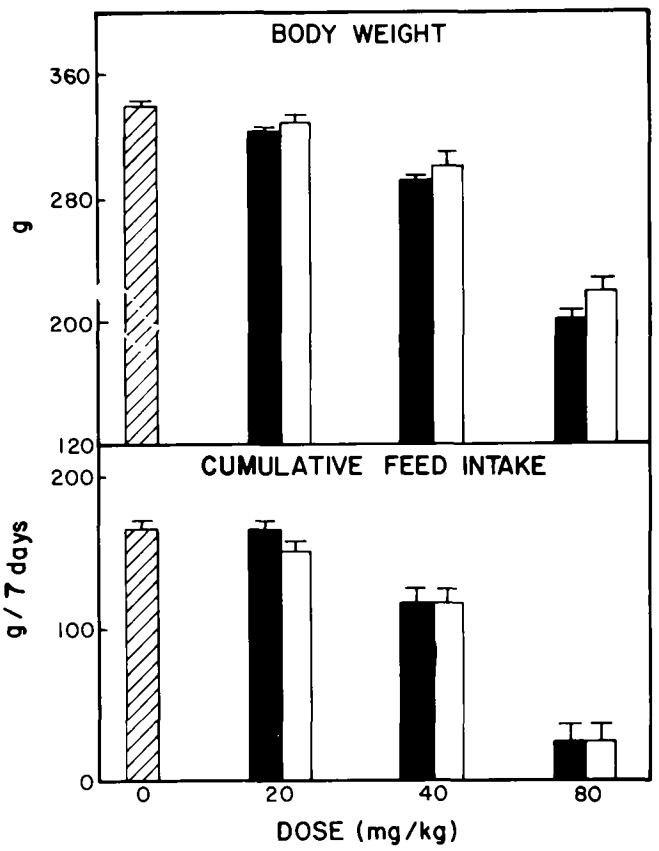

FIG. 1. Body weight and cumulative feed intake in PFDA- (20, 40 or 80 mg/kg) and vehicle-treated rats seven days after treatment. Vehicle-treated rats were either allowed unlimited access to feed (hatched bar) or were pair-fed (open bar) to PFDA-treated rats (closed bar). Each bar and its associated vertical line represent the mean and one S.E.M. (n = 4 for each treatment group).

Carcass. Content of triacylglycerols, whether expressed per g wet wt, carcass, or g fat, was significantly reduced in both PFDA-treated rats and their vehicle-treated, pair-fed counterparts at the 80 mg/kg dose level (Fig. 2). However, PFDA-treated rats at each dose had a higher concentration of triacylglycerols per g wet wt carcass than their pair-fed partners. A reduction in lipid phosphorus per carcass was found in the PFDA-treated rats as well as their vehicle-treated, pair-fed counterparts with increasing dose (Table 1). As a proportion of carcass fat, lipid phosphorus was enriched in the vehicle-treated, pair-fed rats at the 80 mg/kg dose level when compared with either the vehicle-treated group with unlimited access to feed or PFDA-treated rats at the same dose (Table 1). Lipid phosphorus per g fat was reduced in PFDA-treated rats at the 20 and 40 mg/kg dose levels but elevated at 80 mg/kg, in comparison with vehicle-treated rats with unlimited access to feed. With a dose of 40 mg/kg, the concentration of lipid phosphorus in carcass fat of rats receiving PFDA was less than their vehicle-treated, pair-fed partners and this difference was significant (Table 1). The concentration and content of free cholesterol in the carcass did not differ significantly between any of the treatment groups (Table 1). When expressed as a proportion of the carcass fat, free cholesterol was significantly higher in the vehicle-treated rats pair-fed to their PFDA-partners at the 80 mg/kg dose than in other vehicle-treated groups. A similar but less extensive increase was seen in the PFDA-treated rats at the 80 mg/kg dose, and the difference from their vehicle-treated, pair-fed partners was significant (Table 1). An augmentary effect of PFDA on esterified cholesterol concentration and content in the carcass was demonstrated by a significant treatment effect in the two-way ANOVA; however, the difference between PFDA-treated rats and their vehicle-treated, pair-fed partners at each dose level was not significant by pairwise comparison (Table 1).

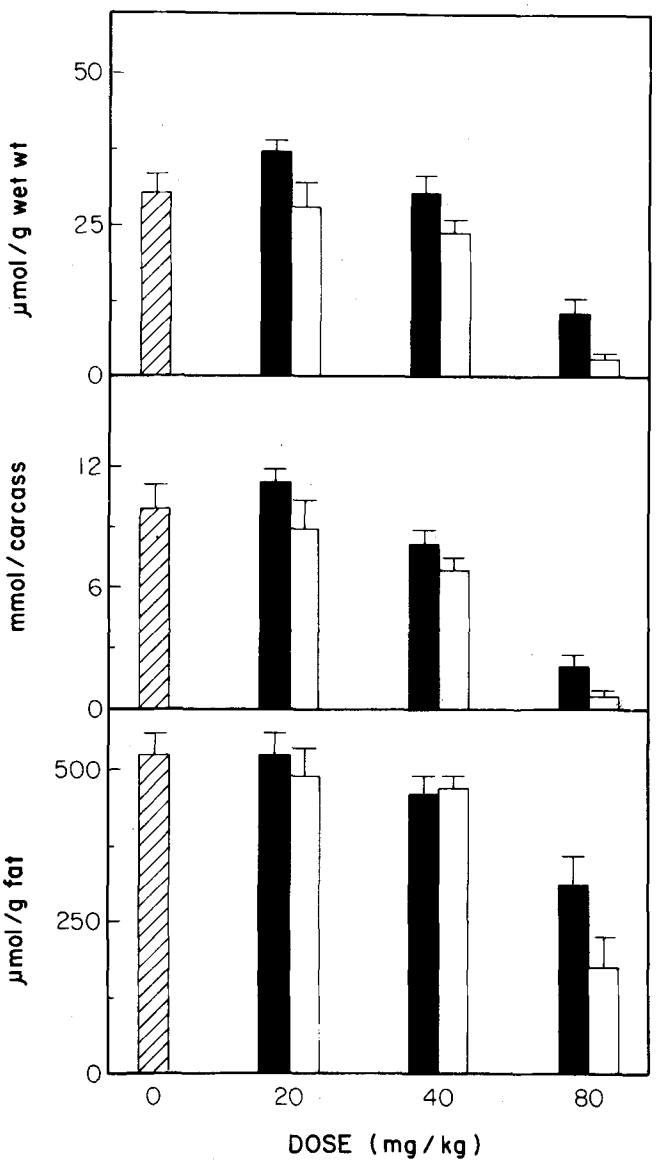

FIG. 2. Effect of PFDA treatment on carcass triacylglycerols in the rat. Vehicle-treated rats were either allowed unlimited access to feed (hatched bar) or were pair-fed (open bar) to PFDA-treated rats (closed bar). Each bar and its associated vertical line represent the mean and one S.E.M. (n = 4 for each treatment group).

Liver. Absolute and relative weight in PFDA-treated animals was significantly greater than in their vehicle-treated, pair-fed partners at all doses examined. When compared with the treatment groups receiving 20 and 40 mg/kg, the absolute increase in liver mass was significantly less, but relative liver weight was maintained in PFDA-treated rats at the 80 mg/kg dose level (Table 2). At the 80 mg/kg dose level, liver mass expressed on an absolute and relative basis, was reduced in vehicle-treated animals pair-fed to PFDA-treated rats (Table 2).

Hepatic DNA concentration was significantly higher in vehicle-treated rats pair-fed to those receiving 80 mg/kg PFDA when compared to the other vehicle-treated groups (Table 2). The concentration of DNA in the livers of PFDA-treated rats was lower than in their vehicle-treated pair-fed counterparts, and this difference was significant at the 20 and 80 mg/kg dose levels (Table 2). The DNA content per liver was reduced in a dose-related fashion in vehicle-treated rats, whereas it was maintained in the animals receiving PFDA at all doses. The difference in total DNA per liver between PFDA-treated rats and their vehicle-treated, pair-fed partners at the 40 and 80 mg/kg dose levels was significant (Table 2). Hepatic protein concentration was higher in vehicle-treated, pair-fed groups than in their PFDA-treated partners, and this difference was significant at the 80 mg/kg dose level (Table 2). At the 80 mg/kg dose level, total protein per liver was reduced significantly in rats receiving PFDA as well as their vehicle-treated, pair-fed partners. When hepatic protein was expressed per mg DNA, no statistically significant differences were detected between any of the treatment groups (Table 2).

Hepatic concentration of lipid phosphorus and free cholesterol was similar in all treatment groups (Table 3). When expressed per liver, a significant reduction in the content of lipid phosphorus and free cholesterol was found in vehicle-treated, pair-fed rats at the 80 mg/kg dose level, when compared with either the other vehicle-treated groups or those receiving PFDA. On a cellular basis (per mg DNA), lipid phosphorus but not free cholesterol was influenced significantly by dose with a decline at the 80 mg/kg dose level (Table 3).

TABLE 1

Effects of PFDA on Carcass Lipid Composition of Adult Male Rats[a]

Dose mg/kg	Treatment	Lipid phosphorus			Cholesterol					
					Free			Esterified		
		μmol/g wet wt	mmol/carcass	μmol/g fat	μmol/g wet wt	mmol/carcass	μmol/g fat	μmol/g wet wt	mmol/carcass	μmol/g fat
0	Unlimited-fed	10.1 ± 0.6^{e}	3.26 ± 0.16^{b,e}	179 ± 25^{b,c,e}	3.78 ± 1.21	1.19 ± 0.36	70.1 ± 31.0^{b,c,e}	0.75 ± 0.19	0.24 ± 0.06	13.3 ± 4.0
20	PFDA	8.95 ± 0.54	2.72 ± 0.15^{b,c}	127 ± 8^{b}	2.91 ± 0.12	0.89 ± 0.03	41.3 ± 2.1^{b}	2.07 ± 0.54	0.63 ± 0.17	29.3 ± 7.7
	Pair-fed	8.14 ± 0.38^{e,f}	2.50 ± 0.11^{f}	143 ± 11^{e}	3.05 ± 0.10	0.93 ± 0.01	53.6 ± 4.5^{e}	0.81 ± 0.16	0.25 ± 0.05	14.1 ± 2.5
40	PFDA	8.03 ± 0.79	2.17 ± 0.22^{c,d}	118 ± 13^{b,*}	3.02 ± 0.07	0.82 ± 0.03	45.9 ± 2.4^{b,c}	1.18 ± 0.28	0.32 ± 0.08	17.9 ± 4.3
	Pair-fed	8.25 ± 0.54^{e,f}	2.33 ± 0.19^{f}	161 ± 8^{e}	3.09 ± 0.18	0.85 ± 0.07	59.2 ± 5.1^{e}	0.86 ± 0.20	0.25 ± 0.06	17.0 ± 4.1
80	PFDA	8.89 ± 0.43	1.70 ± 0.08^{d}	283 ± 51^{c}	3.55 ± 0.27	0.68 ± 0.06	110 ± 16^{c,*}	1.17 ± 0.60	0.32 ± 0.11	60.7 ± 26.4
	Pair-fed	7.37 ± 0.63^{f}	1.53 ± 0.13^{g}	413 ± 14^{f}	3.46 ± 0.24	0.72 ± 0.04	194 ± 10^{f}	0.82 ± 0.28	0.17 ± 0.07	49.0 ± 18.0

[a]Values are reported as the mean and S.E.M. for three to four rats.

Mean values in a column not followed by the same superscript (b, c, d) are significantly different from other dose levels within the PFDA treatment group using Scheffé's multiple comparison method ($p < 0.05$). The unlimited-fed group serves as the zero-dose level.

Mean values in a column not followed by the same superscript (e, f, g) are significantly different from other dose levels within the pair-fed treatment group using Scheffé's multiple comparison method ($p < 0.05$). The unlimited-fed group serves as the zero-dose level.

*Difference from the respective pair-fed group is significant ($p < 0.05$).

TABLE 2

Liver Mass and Hepatic Content of DNA and Protein in Male Rats Seven Days Following PFDA Treatment[a]

Dose mg/kg	Treatment	Liver mass		DNA		Protein		
		Absolute g wet wt	Relative %, body wt	mg/g wet wt	mg/liver	mg/g wet wt	g/liver	mg/mg DNA
0	Unlimited-fed	13.0 ± 0.3^{b,c,d}	3.78 ± 0.04^{b,d}	2.12 ± 0.09^{b,c,d}	27.4 ± 1.3^{d}	134 ± 13	1.74 ± 0.20	64 ± 8
20	PFDA	15.7 ± 0.5^{b,*}	4.69 ± 0.07^{c,*}	1.92 ± 0.04^{b,*}	30.2 ± 0.8	113 ± 4	1.78 ± 0.10	59 ± 2
	Pair-fed	12.2 ± 0.2^{d}	3.59 ± 0.07^{d}	2.20 ± 0.10^{d}	26.8 ± 1.3^{d}	137 ± 21	1.68 ± 0.27	64 ± 12
40	PFDA	15.3 ± 0.3^{b,*}	5.09 ± 0.07^{c,*}	1.98 ± 0.09^{b}	30.3 ± 1.3*	118 ± 9	1.80 ± 0.14	60 ± 7
	Pair-fed	11.5 ± 1.1^{d}	3.54 ± 0.28^{d}	2.27 ± 0.16^{d}	25.6 ± 0.8^{d,e}	135 ± 16	1.58 ± 0.28	62 ± 10
80	PFDA	12.3 ± 1.1^{c,*}	5.17 ± 0.20^{c,*}	2.40 ± 0.11^{c,*}	29.4 ± 2.1*	101 ± 2*	1.24 ± 0.10	42 ± 1
	Pair-fed	5.6 ± 1.1^{e}	2.23 ± 0.31^{e}	3.00 ± 0.17^{e}	16.9 ± 3.6^{e}	158 ± 13	0.91 ± 0.24	53 ± 3

[a]Values are reported as the mean and S.E.M. for four rats.

Mean values in a column not followed by the same superscript (b, c) are significantly different from other dose levels within the PFDA treatment group using Scheffé's multiple comparison method ($p < 0.05$). The unlimited-fed group serves as the zero-dose level.

Mean values in a column not followed by the same superscript (d, e) are significantly different from other dose levels within the pair-fed treatment group using Scheffé's multiple comparison method ($p < 0.05$). The unlimited-fed group serves as the zero-dose level.

*Difference from the respective pair-fed group is significant ($p < 0.05$).

TABLE 3

Liver Lipid Phosphorus and Free Cholesterol in Male Rats Seven Days After PFDA Treatment[a]

Dose mg/kg	Treatment	Lipid phosphorus			Free cholesterol		
		μmol/g wet wt	μmol/liver	μmol/mg DNA	μmol/g wet wt	μmol/liver	μmol/mg DNA
0	Unlimited-fed	29.1 ± 1.3	377 ± 23[b]	13.9 ± 1.0	3.10 ± 0.15	39.9 ± 1.8[b]	1.46 ± 0.05
20	PFDA	33.9 ± 5.4	527 ± 77	17.6 ± 2.7	3.21 ± 0.43	51.5 ± 5.9	1.49 ± 0.14
	Pair-fed	31.7 ± 1.6	392 ± 27[b]	14.6 ± 1.2	3.39 ± 0.16	40.9 ± 1.0[b]	1.51 ± 0.14
40	PFDA	28.2 ± 1.4	431 ± 16	14.3 ± 0.8	2.81 ± 0.23	43.8 ± 4.0	1.46 ± 0.04
	Pair-fed	35.4 ± 3.6	417 ± 83[b]	16.1 ± 2.8	3.03 ± 0.12	35.0 ± 4.1[b,c]	1.36 ± 0.12
80	PFDA	28.2 ± 0.4	347 ± 31*	11.8 ± 0.4	4.10 ± 0.74	49.7 ± 7.9*	1.71 ± 0.29
	Pair-fed	27.4 ± 2.1	152 ± 30[c]	9.2 ± 0.8	3.97 ± 0.44	21.9 ± 4.6[c]	1.34 ± 0.17

[a]Values are reported as the mean and S.E.M. for three to four rats.

Mean values in a column not followed by the same superscript (b, c) are significantly different from other dose levels in the pair-fed treatment group using Scheffé's multiple comparison method ($p < 0.05$). The unlimited-fed group serves as the zero-dose level.

*Difference from the respective pair-fed group is significant ($p < 0.05$).

TABLE 4

Plasma Lipid Composition of Male Rats Seven Days Following PFDA Treatment[a]

Dose mg/kg	Treatment	Free fatty acids	3-Hydroxybutyrate	Triacylglycerols	Lipid phosphorus	Cholesterol	
						Free	Esterified
0	Unlimited-fed	0.29 ± 0.03	0.10 ± 0.01	0.33 ± 0.06[b,c]	1.27 ± 0.08[b,e]	0.45 ± 0.06	1.18 ± 0.19[e]
20	PFDA	0.41 ± 0.05	0.14 ± 0.03	0.21 ± 0.03[b,c]	0.91 ± 0.04[c]	0.37 ± 0.02*	0.62 ± 0.09*
	Pair-fed	0.30 ± 0.02	0.17 ± 0.04	0.28 ± 0.03	1.08 ± 0.04[e]	0.46 ± 0.02	0.88 ± 0.05[e,f]
40	PFDA	0.32 ± 0.06	0.08 ± 0.03	0.37 ± 0.03[b]	0.97 ± 0.02[c]	0.41 ± 0.05	0.56 ± 0.06*
	Pair-fed	0.36 ± 0.07	0.23 ± 0.09	0.23 ± 0.06	0.93 ± 0.08[e,f]	0.43 ± 0.03	0.94 ± 0.03[e,f]
80	PFDA	0.31 ± 0.01	0.21 ± 0.09	0.17 ± 0.03[c]	0.51 ± 0.02[d]	0.48 ± 0.07*	0.73 ± 0.14
	Pair-fed	0.24 ± 0.08	0.17 ± 0.07	0.17 ± 0.04	0.58 ± 0.12[f]	0.30 ± 0.03	0.54 ± 0.08[f]

[a]Values are reported as the mean and S.E.M. for three to four rats and are expressed as μmol/ml plasma.

Mean values in a column not followed by the same superscript (b, c, d) are significantly different from other dose levels within the PFDA treatment group using Scheffé's multiple comparison method ($p < 0.05$). The unlimited-fed group serves as the zero-dose level.

Mean values in a column not followed by the same superscript (e, f, g) are significantly different from other dose levels within the pair-fed treatment group using Scheffé's multiple comparison method ($p < 0.05$). The unlimited-fed group serves as the zero-dose level.

*Difference from the respective pair-fed group is significant ($p < 0.05$).

PFDA treatment resulted in a dose-related increase in liver triacylglycerols (Fig. 3). When expressed per g wet wt or mg DNA, the increment of hepatic triacylglycerols between PFDA-treated rats and their vehicle-treated, pair-fed counterparts at the 40 and 80 mg/kg dose levels was significant (Fig. 3). The content of triacylglycerols per liver was significantly greater in PFDA-treated rats than their vehicle-treated, pair-fed partners at all doses examined. However, liver triacylglycerols expressed per g wet wt, liver or mg DNA, were diminished in the vehicle-treated rats pair-fed to those receiving 80 mg/kg PFDA when compared with the other vehicle-treated groups (Fig. 3).

The response of hepatic esterified cholesterol to PFDA treatment was similar but less pronounced than that of liver triacylglycerols. A dose-related increase in liver esterified cholesterol was found in PFDA-treated rats (Fig. 4). The difference in hepatic esterified cholesterol between rats receiving PFDA and their pair-fed partners was significant at the 80 mg/kg dose level, whether expressed per g wet wt, liver or mg DNA. Hepatic esterified cholesterol also was elevated in PFDA-treated rats at the 40 mg/kg dose, but the difference from their vehicle-treated, pair-fed partners was statistically significant only when expressed per liver.

Plasma. The plasma level of free fatty acids and 3-hydroxybutyrate was similar in all treatment groups (Table 4). The concentration of triacylglycerols and lipid phosphorus in plasma decreased in a dose-related fashion in both PFDA-treated and pair-fed groups, but a significant difference between treatments was not detected by two-way ANOVA (Table 4). With a dose of 20 mg/kg, the plasma concentration of free cholesterol was lower in rats receiving PFDA than in their vehicle-treated, pair-fed partners (Table 4). At the 80 mg/kg dose level, the concentration of free cholesterol was higher in the plasma of PFDA-treated rats than their pair-fed counterparts, and the difference was significant. This dissimilarity resulted from a decrease in the plasma level of free cholesterol in the vehicle-treated, pair-fed group (Table 4). Also, the vehicle-treated rats pair-fed to their partners receiving 80 mg/kg PFDA had a concentration of

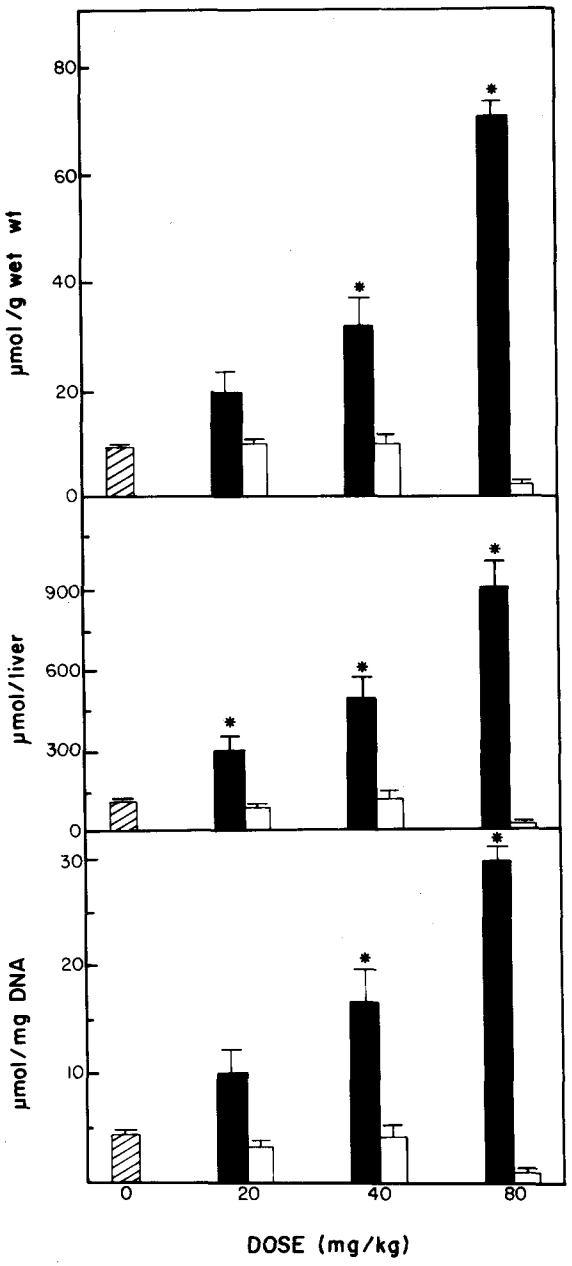

FIG. 3. Liver triacylglycerols in rats seven days after PFDA treatment. Vehicle-treated rats were either allowed unlimited access to feed (hatched bar) or were pair-fed (open bar) to PFDA-treated rats (closed bar). Each bar and its associated vertical line represent the mean and one S.E.M. (n = 4 for each treatment group). *Difference from the respective pair-fed group is significant ($p < 0.05$).

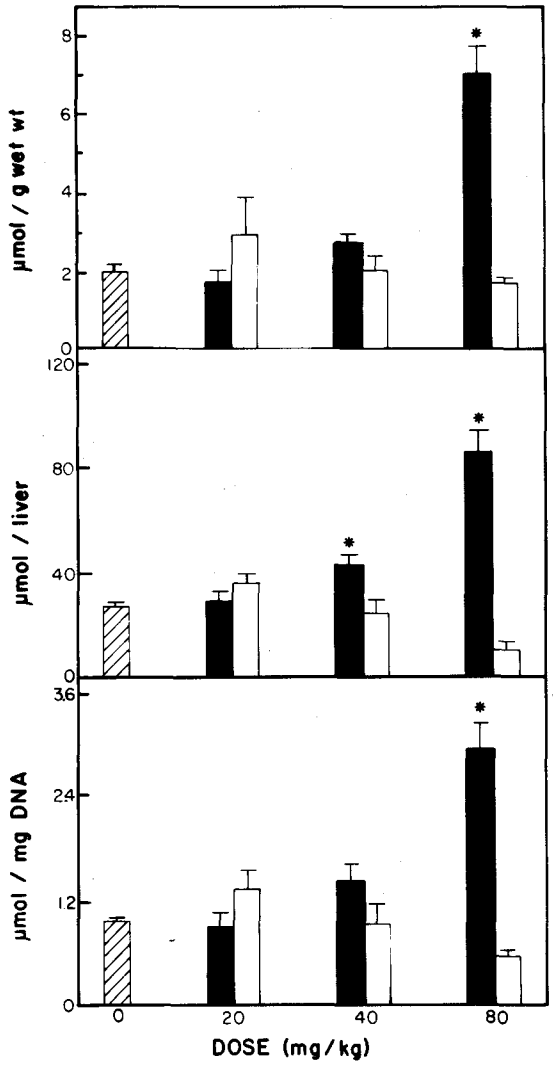

FIG. 4. Liver esterified cholesterol in rats seven days after PFDA treatment. Vehicle-treated rats were either allowed unlimited access to feed (hatched bar) or were pair-fed (open bar) to PFDA-treated rats (closed bar). Each bar and its associated vertical line represent the mean and one S.E.M. (n = 4 for each treatment group). *Difference from the respective pair-fed group is significant ($p < 0.05$).

esterified cholesterol in plasma that was lower than the other vehicle-treated groups, whether pair-fed or with unlimited access to feed (Table 4). In PFDA-treated rats, regardless of dose, the plasma level of esterified cholesterol was lower than that of the vehicle-treated group with unlimited access to feed (Table 4). At the 20 and 40 mg/kg dose levels, the concentration of esterified cholesterol in plasma also was significantly lower in PFDA-treated rats than their vehicle-treated, pair-fed partners.

DISCUSSION

Following a single intraperitoneal injection of PFDA, alterations in lipid metabolism of the adult male Sprague-Dawley rat were investigated seven days after dosing. Because the administration of PFDA causes a dose-dependent reduction in feed (caloric) intake (3–5), it was important to take this into account in the interpretation of the data through the use of a vehicle-treated rat, pair-fed to each one receiving PFDA. Thus, the primary effects of PFDA on lipid metabolism could be distinguished both qualitatively and quantitatively from those secondary to hypophagia.

A dose-related decrease in both body and carcass weight, associated with a diminution in caloric intake, was seen in rats receiving PFDA as well as their vehicle-treated, pair-fed counterparts. Reduction in the carcass content of lipid phosphorus (phospholipid) and free

cholesterol in rats treated with PFDA would seem to be consequent to hypophagia and resulting weight loss, as the appropriate vehicle-treated, pair-fed animals exhibited the same attenuation. Carcass triacylglycerols decreased in a dose-related fashion in both PFDA-treated rats and their pair-fed partners, due to the negative caloric balance, i.e., feed intake. Yet, at every dose level examined (20–80 mg/kg), PFDA-treated rats were found to have a concentration of triacylglycerols in their carcass greater than that of their vehicle-treated, pair-fed counterparts. A similar increment in carcass ether-extractable fat also was detected between PFDA- and vehicle-treated rats (5). Differences in carcass lipid composition between the various treatment groups also were compared by expressing each lipid class as a proportion of ether-extractable fat. At the highest dose (80 mg/kg) examined, the loss of ether-extractable fat in the vehicle-treated rats pair-fed to their partners receiving PFDA was accompanied by an elevation in the proportion of lipid phosphorus and free cholesterol with a concomitant reduction in triacylglycerols. In rats treated with 80 mg/kg PFDA, a similar pattern but less extensive shift in the composition of the ether-extractable fat was found. This was the result of the higher concentration of triacylglycerols found in the carcasses of PFDA-treated rats than their pair-fed partners.

Major shifts in the relative percentages of fatty acids (3) and an increase in the activity of peroxisomal fatty acyl-CoA oxidase (25,26) in the rat liver, following the administration of PFDA would suggest that the perfluorinated fatty acid probably interacts with hepatic lipid metabolism. So that the changes in the concentrations of the various lipids could be compared, differences in hepatic cellularity between the different treatment groups had to be considered. In PFDA-treated rats, liver mass (absolute and relative) increased when compared with that of appropriate vehicle-treated, pair-fed animals. At each dose examined (20–80 mg/kg), an increase in DNA concentration (indicative of a smaller hepatocyte) in vehicle-treated, pair-fed rats was detected when contrasted with their partners receiving PFDA. Reduction in the DNA content per liver (indicating a loss of cells) also was detected in vehicle-treated, pair-fed rats at the 40 and 80 mg/kg dose levels, seven days post-treatment. Content of phospholipid and free cholesterol per total liver was reduced in the vehicle-treated rats pair-fed to those receiving 80 mg/kg PFDA. Corresponding decreases were reported in feed-restricted animals (27–29). A similar decrease in liver content of phospholipids and free cholesterol was not found in the PFDA-treated rats. In spite of this apparent difference between the PFDA-treated animals and their vehicle-treated, pair-fed counterparts, phospholipid and free cholesterol per cell was not different among the various treatment groups when changes in cellularity were taken into account.

Even though there was no effect of PFDA on the amount of phospholipid and free cholesterol per cell, a marked increase in the content of triacylglycerols per liver was detected. The accumulation of hepatic triacylglycerols can occur for a number of reasons, including increased fatty acid load (endogenous and exogenous), decreased fatty acid oxidation, increased synthesis and/or reduced breakdown of triacylglycerols, and a decreased export as very low density lipoproteins (30). Regulation of triacylglycerol synthesis is complex and not fully understood. However, the availability of fatty acids plays a major role in the esterification pathway (31). Free fatty acids in the circulation are taken up by the liver in a concentration-dependent manner (32,33). The level of free fatty acids in the plasma of PFDA-treated rats was similar to those receiving vehicle, whether pair-fed or with unlimited access to feed. However, this determination was an indication of the concentration of free fatty acids at a single time point, seven days after dosing. The increase in the relative percentage of oleic acid in the lipids of livers from PFDA-treated rats (3,4) would suggest that the peripheral fat stores (34) were the source of long chain fatty acyl-CoA's for the synthesis of triacylglycerols. Augmentation in the hepatic content of triacylglycerols already was detected at the lowest dose (20 mg/kg) of PFDA examined. It is possible that PFDA, similar to other carboxylic acids such as 2-bromooctanoic acid and 4-pentenoic acid (35–38), does inhibit fatty acid oxidation, thereby diverting fatty acids from oxidation toward esterification. As with triacylglycerols, a similar but less pronounced augmentation in the content of esterified cholesterol was detected in the PFDA-treated rats. While cholesterol ester synthesis appears to depend on the supply of unesterified cholesterol, moderate increases in long chain fatty acyl-CoA's also can stimulate this esterification pathway (39). Thus, it is conceivable that in PFDA-treated rats this pathway becomes available for the removal of excess long chain fatty acyl-CoA's in addition to esterification as triacylglycerols. However, synthesis of triacylglycerols would appear to be much more sensitive to increases in the concentration of long chain fatty acyl-CoA's. The hepatic capacity for formation of triacylglycerols would seem to be much greater than cholesterol esters.

PFDA has been found to be very persistent and can be detected in the liver of male Fischer-344 rats 30 days after a single intraperitoneal dose of 50 mg/kg (40). If PFDA and/or its activated derivatives (CoA or carnitine esters) were able to partially inhibit hepatic oxidation of fatty acids (35–38), one would expect triacylglycerols to increase. Thus, the impairment of fatty acid oxidation by PFDA could then result in a diminished NADH supply for ATP generation. This has been found in the presence of bromooctanoate, a known inhibitor of fatty acid oxidation (41). The resultant lowering of hepatic ATP concentration could in turn lead to a decrease in protein synthesis including that of apolipoproteins, e.g., apo-B needed for export of triacylglycerols from the liver (30,42). Even though hepatic DNA, phospholipid and free cholesterol content were maintained in PFDA-treated rats at 80 mg/kg, the amount of protein per hepatocyte was decreased. The increase in the activities of hepatic malic enzyme and L-glycerol-6-phosphate dehydrogenase found at lower doses of PFDA (20 and 40 mg/kg) was reduced or obliterated, respectively, in rats receiving 80 mg/kg (43). Thus, a potential decreased hepatic synthesis of apolipoproteins limiting very low density lipoprotein synthesis could contribute to the accumulation of triacylglycerols found in PFDA-treated rats at the highest dose examined (80 mg/kg). Furthermore, plasma triacylglycerols were not elevated in PFDA-treated rats, despite the hepatic augmentation of esterified compounds. The increase in hepatic content of triacylglycerols and esterified

cholesterol would suggest diversion of long chain fatty acyl-CoA's from oxidation toward esterification in livers of PFDA-treated rats.

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REFERENCES

1. Guenthner, R.A., and Vietor, L.M. (1962) *Ind. Eng. Chem. Prod. Res. Dev. 1,* 165-169.
2. Shinoda, K., and Nomura, T. (1980) *J. Phys. Chem. 84,* 365-369.
3. Olson, C.T., and Andersen, M.E. (1983) *Toxicol. Appl. Pharmacol. 70,* 362-372.
4. George, M.E., and Andersen, M.E. (1986) *Toxicol. Appl. Pharmacol. 85,* 169-180.
5. Van Rafelghem, M.J., Noren, C.W., Menahan, L.A., and Peterson, R.E., *Toxicol. Lett. 40,* 57-69.
6. Kelling, C.K., Van Rafelghem, M.J., Drake, R.L., Menahan, L.A., and Peterson, R.E. (1986) *J. Biochem. Toxicol. 1*(3), 23-37.
7. Wollenberger, A., Ristau, O., and Schoffa, G. (1960) *Pflügers Arch. 270,* 399-412.
8. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
9. Laurell, S. (1966) *Scand. J. Clin. Lab. Invest. 18,* 668-672.
10. Christie, W.W. (1973) in *Lipid Analysis,* pp. 222-223, Pergamon Press, New York.
11. Zilversmit, D.B., and Davis, A.K. (1950) *J. Lab. Clin. Med. 35,* 155-160.
12. Deacon, A.C., and Dawson, P.J.G. (1979) *Clin. Chem. 25,* 976-984.
13. Salè, F.O., Marchesini, S., Fishman, P.H., and Berra, B. (1984) *Anal. Biochem. 142,* 347-350.
14. Mellanby, J., and Williamson, D.H. (1974) in *Methods of Enzymatic Analysis* (H.U. Bergmeyer, ed.) pp. 1840-1843, Academic Press, New York.
15. Williamson, D.H., and Mellanby, J. (1974) in *Methods of Enzymatic Analysis* (H.U. Bergmeyer, ed.) pp. 1836-1839, Academic Press, New York.
16. Hron, T.W., and Menahan, L.A. (1981) *J. Lipid Res. 22,* 377-381.
17. Livingston, J.N., Cuatrecasas, P., and Lockwood, D.H. (1974) *J. Lipid Res. 15,* 26-32.
18. Richards, G.M. (1974) *Anal. Biochem. 57,* 369-376.
19. Bradford, M.M. (1976) *Anal. Biochem. 72,* 248-254.
20. Milliken, G.A., and Johnson, D.E. (1984) in *Analysis of Messy Data. Volume I: Designed Experiments,* pp. 35-36, 146-155, Van Nostrand Reinhold Co., New York.
21. Neter, J., and Wasserman, W. (1974) *Applied Linear Statistical Methods,* Richard D. Irwin, Homewood, IL.
22. SAS User's Guide: Statistics (1985) SAS Institute Inc., Cary, NC.
23. Owen, J.S., and McIntyre, N. (1982) *Trends Biochem. Sci. 7,* 95-98.
24. Hollanders, B., Mougin, A., N'Diaye, F., Hentz, E., Aude, X., and Girard, A. (1986) *Comp. Biochem. Physiol. 84B,* 83-98.
25. Van Rafelghem, M.J., Andersen, M.E., Lane, S., Luking, S., and Harrison, E.H. (1984) *Toxicologist 4,* 174.
26. Harrison, E.H., Lane, J.S., Luking, S., Van Rafelghem, M.J., and Andersen, M.E. (1988) *Lipids 23,* 115-119.
27. Freedland, R.A. (1967) *J. Nutr. 91,* 489-495.
28. Herrera, E., and Freinkel (1968) *Biochim. Biophys. Acta 170,* 244-253.
29. Weigand, W., Hannappel, E., and Brand, K. (1980) *J. Nutr. 110,* 669-674.
30. Dianzani, M.U. (1979) in *Toxic Injury of the Liver. Part A* (Farber, E., and Fisher, M.M., eds.) pp. 281-331, Marcel Dekker, Inc., New York.
31. Zammit, V.A. (1984) *Prog. Lipid Res. 23,* 39-67.
32. Van Harken, D.R., Dixon, C.W., and Heimberg, M. (1969) *J. Biol. Chem. 224,* 2278-2285.
33. Goreski, C.A., Daly, D.S., Mishkin, S., and Arias, I.M. (1978) *Am. J. Physiol. 234*(6), E542-E553.
34. Kohout, M., Braun, T., and Mihalec, C. (1965) *Physiol. Bohemoslov. 14,* 460-465.
35. Raaka, B.M., and Lowenstein, J.M. (1979) *J. Biol. Chem. 254,* 3303-3310.
36. Raaka, B.M., and Lowenstein, J.M. (1979) *J. Biol. Chem. 254,* 6755-6762.
37. Osmundsen, H., and Sherratt, H.S.A. (1978) *Biochem. Soc. Trans. 6,* 84-88.
38. Schulz, H. (1987) *Life Sci. 40,* 1443-1449.
39. Spector, A.A., Mathur, S.N., and Kaduce, T.L. (1979) *Prog. Lipid Res. 18,* 31-53.
40. George, M.E., and Andersen, M.E. (1986) *Toxicologist 6,* 315.
41. Danis, M., Kauffman, F.C., Evans, R.K., and Thurman, R.G. (1981) *J. Pharmacol. Exp. Ther. 219,* 383-388.
42. Lombardi, B. (1965) *Fed. Proc. 24,* 1200-1205.
43. Kelling, C.K., Van Rafelghem, M.J., Menahan, L.A., and Peterson, R.E. (1987) *Biochem. Pharmacol. 36,* 1337-1344.

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Substituted Fatty Acids in the Leaves of Some Higher Plants

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Substituted (hydroxy-, epoxy- and dicarboxy) fatty acids have been analyzed in the needles of two gymnosperms *(Pinus sylvestris* and *Juniperus communis)* and the leaves of four angiosperms *(Betula verrucosa, Populus nigra, Quercus petraea* and *Corylus avellana)* growing in the Lake Léman basin. The differences in the distributions of the acids that have been analyzed are greater between the various species than between the spring and autumn samples of a given species. Moreover, the isomeric composition of the 8,16-, 9,16- and 10,16-dihydroxypalmitic acid mixture is specific for each species. *Lipids 23,* 679–681 (1988).

Studies on plant fatty acids have long been restricted mainly to the lipids of the storage organs such as seeds and tubers. The fatty acids of epicuticular waxes, which play an important role in preserving a correct water balance for the plants (1,2), also have been studied for a long time, as well as the fatty acids present in cutin and suberin, because of their protective action against pathogenic fungi and microbes (3). During the last decade, the rapid development of organic geochemistry led to an increased interest in a better knowledge of the composition and distribution of fatty acids in higher plants; although they are more or less easily biodegradable, part of the fatty acids present in plant wax esters and in cutin are incorporated into lacustrine and marine sediments, where they remain unchanged for long periods (4,5). Thus, the composition and the distribution of fatty acids extracted from recent and ancient sediments give information on the sources of the sedimentary organic matter.

In this study, we have analyzed the substituted fatty acids in the whole leaves and needles of *Pinus sylvestris* (pine), *Juniperus communis* (juniper), *Betula verrucosa* (birch), *Populus nigra* (poplar), *Quercus petraea* (oak) and *Corylus avellana* (hazel). The choice of these six species was based on the results of a palynological study of sediments from the Lake Léman basin that showed, since some 14,000 years ago, periods with a predominance of one or several of these species had occurred (6). Our objective was to examine if there existed significant differences between the fatty acid compositions that could be exploited in the geochemical/environmental correlation study that we currently are conducting on the sediments of the Lake Léman Basin (7,8).

EXPERIMENTAL

Immediately after collection, the samples were lyophilized to constant weight. For each plant, between 0.1 and 0.2 g of dried leaves or needles were finely crushed and hydrolyzed during 12 hr by refluxing them in a solution of 10% potassium hydroxide in methanol. Qualitative and quantitative analyses of the hydrolysis products were carried out using the analytical techniques described for sedimentary acids (7,8). Briefly, the hydrolyzate was acidified, and the acidic fraction was isolated by chromatography on a silica gel column (200 × 12 mm, 70-230 mesh, 10% KOH). After methylation with BF_3-MeOH, the acidic fraction was again submitted to chromatography on a silica gel column (300 × 6 mm, same packing as before). The monocarboxylic acid methyl esters were eluted with a mixture of hexane/CH_2Cl_2 (3:1, v/v) and those of the hydroxy- and dicarboxylic acids with a mixture of CH_2Cl_2/EtOAc (7:3, v/v). The recoveries of the hydroxy acids from the columns have been tested through the addition of known quantities of 2-hydroxytetradecanoic and 3-hydroxyheneicosanoic acids and were found to be quantitative.

The hydroxy acid fractions were treated with bis(trimethylsilyl)trifluoroacetamid (BSTFA) to form the trimethylsilyl ethers (TMS). The gas chromatographic (GC) analyses were performed on a 25 m × 0.32 mm OV—73 capillary column using helium as the carrier gas (2.5 ml/min), a splitless injection mode and a flame ionization detector (FID). The column temperature was programmed from 60 to 150 C at 10 C/min and from 150 C to 290 C at 4 C/min. The gas chromatography-mass spectrometry (GC/MS) analyses were carried out on a quadrupole instrument operating at a nominal ionizing potential of 70 eV and an ion source temperature of 250 C. The chromatographic conditions were similar to those used for the GC runs.

The compounds were identified by comparing the GC equivalent chain length values (ECL) and mass spectrometric data with those of authentic samples or data published in the literature (9–11). Quantitative determinations were made by comparing the areas of the GC-FID peaks with that of a known quantity of 2-hydroxytetradecanoic acid used as an internal standard, assuming the same response factors for all the components. The isomeric compositions of the dihydroxyhexadecanoic and hydroxy-1,16-hexadecanedioic acids were determined by measuring the abundances of the characteristic ions arising from the cleavage α to the secondary TMS ether groups (9). 9,10-Epoxy-18-hydroxyoctadecanoic acid was analyzed as a GC coeluting mixture of 9-methoxy-10,18-dihydroxy- and 10-methoxy-9,18-dihydroxyoctadecanoic acids formed during the saponification of the samples (12).

RESULTS AND DISCUSSION

Table 1 shows the amounts and the compositions of the substituted fatty acids extracted from the spring and autumn leaves and needles of the six species that were studied. In all the species, the major acids are the positional isomers of dihydroxypalmitic acid. With the exception of the α-hydroxyacids, all the acids we have found are typical of those present in cutin (3); α-hydroxyacids usually are not found in cutin and have seldom been reported as constituents of the intercellular lipids of higher plants (13). In the samples we have analyzed, they

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Abbreviations: BSTFA, bis(trimethylsilyl)trifluoroacetamid; TMS, trimethylethers; ECL, equivalent chain length values; FID, flame ionization detector; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry.

TABLE 1

Hydroxy-, Dicarboxy- and Epoxy Acid Compositions (%) of the Lipids from the Leaves of Six Higher Plant Species

	B. verrucosa		*Q. petraea*		*C. avellana*		*P. nigra*		*P. sylvestris*		*J. communis*	
ECL Acid	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall
1573 ω-OH-C12:0	—	—	—	—	—	—	—	—	18.8	23.3	14.3	8.8
1770 ω-OH-C14:0	—	—	—	—	—	—	—	—	11.7	11.4	6.2	3.8
1810 α-OH-C16:0	0.5	0.3	2.4	0.8	1.1	0.9	1.4	0.4	—	0.1	—	0.1
1929 α,ω-dic-C16:0	0.2	1.3	—	—	1.0	0.9	—	—	—	—	—	0.5
1963 ω-OH-C16:0	4.8	6.7	3.5	2.1	10.4	12.0	6.2	4.1	22.3	32.9	25.3	25.5
2033 M-OH-α,ω-dic-C15:0[a]	0.2	0.5	—	0.2	0.6	—	0.6	1.7	1.0	0.8	4.0	0.9
2057 9,15-diOH-C15:0	0.1	0.5	—	0.4	—	—	0.0	0.2	—	—	—	0.4
2109 ω-OH-C18:2	—	—	—	—	24.3	8.4	—	—	—	—	—	—
2132 M-OH-α,ω-dic-C16:0[a] ω-OH-C18:1	2.5	3.9	5.2	34.2	13.9	13.8	2.9	6.3	3.4	2.8	4.3	5.1
2166 M,16-diOH-C16:0[a]	89.0	83.5	66.3	32.1	41.2	58.5	77.6	83.7	31.1	24.4	45.9	54.7
2392 α-OH-C22:0	0.5	0.3	3.0	1.7	1.7	1.0	4.5	0.9	—	—	—	—
2425 9,10-epoxy-18-OH-C18:0	0.2	0.3	9.3	26.1	0.2	1.1	—	—	11.7	4.3	—	—
2495 α-OH-C23:0	0.2	0.2	3.3	—	0.5	0.4	1.2	0.7	—	—	—	—
2588 α-OH-C24:0	1.3	1.7	5.3	1.7	3.8	2.4	4.6	1.3	—	0.1	—	0.1
2690 α-OH-C25:0	0.2	0.3	0.6	0.2	0.4	0.3	0.3	0.6	—	—	—	—
2786 α-OH-C26:0	0.3	0.4	1.1	0.4	0.9	0.4	0.8	0.3	—	—	—	—
TOTAL (ppm)[b]	15871	10359	2278	3382	5812	1580	3877	6049	12751	21167	11361	21578

[a]M, midchain substituted (mixture of positional isomers).

[b]ppm : μg/g of freeze-dried leaves.

ECL, equivalent chain length.

are absent from the spring needles of the two conifers but represent as much as 12.8% in the spring leaves of *P. nigra*.

It is only in *Q. petraea* and *C. avellana* that the composition of the fatty acids shows a significant evolution between spring and autumn. The proportions of the dihydroxypalmitic acid isomers sharply decrease in the leaves of the oak, while those of 9,10-epoxy-18-hydroxyoctadecanoic and 18-hydroxyoctadecenoic acids increase, the importance of the latter having been measured by GC-MS.

The differences between the species are remarkable. The two conifers are characterized by their content in ω-hydroxyacids. They both contain ω-hydroxypalmitic acid in higher proportion than the other species and also the $C_{12:0}$ and $C_{14:0}$ homologues, which are totally missing in the four angiosperms. ω-Hydroxyoctadecadienoic acid is found only in *C. avellana* and the 9,10-epoxy-18-hydroxyoctadecanoic acid, which belongs to the major components of *Q. petraea* and *P. sylvestris*, is present in only traces, or even absent, in the other species.

The acids extracted from *B. verrucosa* and *P. nigra* show very similar gas chromatograms, but they differ in the proportions of the 8,16-, 9,16- and 10,16-dihydroxyhexadecanoic acids they contain. These positional isomers, and also the two isomers of the midchain hydroxy-α,ω-hexadecandioic acids listed in Table 2, can indeed not be separated by GC, but the isomeric composition of mixtures of them can be determined using GC-MS (9). Table 2 shows that *P. sylvestris* contains almost exclusively the 9,16-dihydroxy isomer and *J. communis* primarily the 10,16-dihydroxy isomer. The two gymnosperms also completely differ with respect to their relative content in the 7-hydroxy and 8-hydroxy isomers of the midchain hydroxy-α,ω-hexadecandioic acids. Our results are in good agreement with those that already have been published for *P. sylvestris* (10) and *B. verrucosa* (9). We also have examined a mixture of barks and stems from *P. sylvestris* and found the same specificity as in the needles. This suggests that for a given species, the isomeric composition is the same in the whole plant.

In addition to the substituted fatty acids, the polar fractions of all the samples contain phenolic acids, mainly p-coumaric and ferulic acids, which also are known to be components of cutin (12). Moreover, the needles of *P. sylvestris* are characterized by a high content of pinifolic acid (14).

TABLE 2

Positional Isomer Composition (%) of Dihydroxyhexadecanoic (M,16-diOH-C16:0) and Hydroxy-α,ω-dicarboxyhexadecanoic (M-OH-α,ω-dic-C16:0) Acids in the Autumn Leaf Lipids

	M,16-di-OH-C16:0			M-OH-α,ω-dic-C16:0	
	8,16-di-OH	9,16-di-OH	10,16-di-OH	7-OH	8-OH
B. verrucosa	4.4	72.9	22.7	15.3	84.7
Q. petraea	—	8.6	91.4	73.8	26.2
C. avellana	—	27.6	72.4	40.6	59.4
P. nigra	—	12.9	87.1	37.5	62.5
J. communis	—	1.0	99.0	93.4	6.6
P. sylvestris	—	98.2	1.8	1.0	99.0

In conclusion, our results show that using the composition of the hydroxy mono- and dicarboxylic fatty acids for chemical taxonomy is quite feasible with the six species we have analyzed. Each one of them can be unambiguously characterized by the composition of the substituted acids present in its leaves or needles. When present in sediments, these acids thus can give information on the vegetation that existed at the time of their deposition, information that can be correlated with palynological data.

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REFERENCES

1. Eglinton, G., and Hamilton, R.J. (1967) *Science 156,* 1322-1335.
2. Kolattukudy, P.E. (1970) *Lipids 5,* 259-275.
3. Kolattukudy, P.E. (1980) *Science 208,* 990-1000.
4. Haug, P., Schnoes, H.K., and Burlingame, A.L. (1971) *Chem. Geol. 7,* 213-236.
5. Cardoso, J.N., and Eglinton, G. (1983) *Geochim. Cosmochim. Acta 47,* 723-730.
6. Olive, P. (1972) *Revue Géogr. Phys. Géol. Dyn. 14,* 253-264.
7. Mendoza, Y.A., Gülaçar, F.O., and Buchs, A. (1987) *Chem. Geol. 62,* 307-319.
8. Mendoza, Y.A., Gülaçar, F.O., and Buchs, A. (1987) *Chem. Geol. 62,* 321-330.
9. Holloway, P.J., and Deas, A.H.B. (1971) *Phytochemistry 10,* 2781-2785.
10. Hunneman, D.H., and Eglinton, G. (1972) *Phytochemistry 11,* 1989-2001.
11. Eglinton, G., Hunneman, D.H., and McCormick, A. (1968) *Org. Mass Spectrom. 1,* 593-611.
12. Franich, R.A., and Volkman, J.K. (1982) *Phytochemistry 21,* 2687-2689.
13. Kolattukudy, P.E. (1980) in *Biochemistry of Plants* (Stumpf, P.K., ed.) Vol. 4, pp. 571-645, Academic Press, New York.
14. Enzell, C., and Theander, O. (1962) *Acta Chem. Scand. 16,* 607-614.

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The Phospholipid Fatty Acids of the Marine Sponge *Xestospongia muta*

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The rare phospholipid fatty acids 3,7,11-trimethyldodecanoic (*1*), 5,9-hexadecadienoic (*2*) and 12-methyl-hexadecanoic (*3*) were identified in the marine sponge *Xestospongia muta*. Branched fatty acids in *X. muta* accounted for 35% of the total fatty acid mixture. It was observed that the occurrence of the 5,9-hexadecadienoic acid (*2*) coincides with the complete absence of the very long chain fatty acid 5,9-hexacosadienoic. The acid 5,9,19-octacosatrienoic seems to be found in most *Xestospongia* species.
***Lipids 23,* 682–684 (1988).**

Xestospongia muta, commonly known as the barrel sponge, is normally the largest species in volume that would be encountered in the Caribbean (1). It can take many forms, but the most common is a barrel shape with the outer end having a cone-like cavity, hence the name "barrel sponge." This form may reach 1.5 m in height with a diameter nearly as great. The sponge consistency is hard, almost stony, and the outer surface texture is extremely rugged, often buttressed (2).

X. muta has been the source of many unusual sterols and fatty acids. One important sterol, mutasterol(24-methylene-25-ethylcholesterol), was first isolated by Li et al. (3) and was the first example of sterol side-chain bioalkylation at position 25. Schmitz and Gopichand (4) have reported the isolation of a novel dibromo acetylenic acid from *X. muta,* namely the 14,16-dibromo-7,13-15-hexadecatrien-5-ynoic acid, which showed a slight degree of cytotoxicity. Litchfield and Marcantonio examined the related *Xestospongia halichondroides* and found that it contained 13% of the unusually long phospholipid fatty acid, 5,9,19-octacosatrienoic, together with other "demospongic" acids such as the characteristic 5,9-hexacosadienoic (5).

In this work, we studied the phospholipid fatty acids from the marine sponge *X. muta,* and these results are reported.

EXPERIMENTAL METHODS

X. muta was collected near La Parguera, Puerto Rico, during March 1986. The sponge was washed in sea water, carefully cleaned of all nonsponge debris, and cut into small pieces. Immediate extraction with chloroform/methanol (1:1, v/v) yielded the total lipids. The neutral lipids, glycolipids and phospholipids were separated by column chromatography on ammonium hydroxide-treated silicic acid (100–200 mesh) using the procedure of Privett et al. (6). The fatty acyl components of the phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic hydrogen chloride (7) followed by purification on column chromatography eluting with hexane/ether (9:1, v/v). The resulting methyl esters were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Hewlett-Packard 5995A gas chromatograph-mass spectrometer equipped with a 30 m × 0.32 mm fused silica column coated with SE-54. For the location of double bonds, N-acylpyrrolidide derivatives were prepared by direct treatment of the methyl esters with pyrrolidine/acetic acid (10:1, v/v) in a capped vial (24 hr at 100 C), followed by ethereal extraction from the acidified solution and purification by preparative layer chromatography (PLC). Hydrogenations were carried out in 10 ml of absolute methanol and catalytic amounts of platinum oxide (PtO_2). Mass spectrometry results are available upon request. Mass spectral data of important fatty acids for this discussion are presented below.

3,7,11-Trimethyldodecanoic acid methyl ester. MS m/z (rel. int.) 256 (M^+, 8), 225 (3), 213 (5), 171 (19), 143 (13), 139 (6), 111 (15), 102 (13), 101 (87), 97 (24), 85 (11), 83 (16), 75 (24), 74 (100), 71 (14), 69 (38), 59 (26), 57 (25), 56 (12), 55 (39).

12-Methylhexadecanoic acid methyl ester. MS m/z (rel. int.) 284 (M^+, 17), 255 (2), 241 (9), 227 (2), 199 (7), 185 (25), 171 (6), 157 (14), 143 (47), 130 (18), 129 (17), 101 (15), 97 (20), 87 (69), 83 (23), 75 (27), 74 (100), 57 (47).

12-Methylhexadecanoic acid pyrrolidide. MS m/z (rel. int.) 323 (M^+, 3), 308 (1), 294 (3), 280 (1), 266 (1), 238 (1), 224 (0.5), 210 (0.3), 196 (0.2), 149 (4), 126 (12), 113 (100), 98 (11), 81 (21), 69 (31), 55 (27).

5,9,19-Octacosatrienoic acid methyl ester. MS m/z (rel. int.) 432 (M^+, 1), 207 (3), 193 (4), 181 (5), 180 (8), 167 (4), 163 (5), 150 (10), 149 (11), 140 (22), 136 (13), 135 (15), 123 (11), 121 (14), 110 (21), 109 (42), 108 (12), 99 (20), 95 (26), 94 (24), 93 (11), 85 (12), 81 (100), 74 (15), 67 (56), 55 (80).

RESULTS

The results presented in Table 1 show that *X. muta* contains many branched fatty acids. In fact, branched fatty acids accounted for 35% of the total phospholipid fatty acid mixture. Interesting was the presence of a fatty acid methyl ester with a molecular weight of 256. This fatty acid was inert upon catalytic hydrogenation, thus excluding any possible unsaturation. Moreover, a molecular weight of 256 provides for 15 carbons in the chain but this acid presented an extremely low equivalent chain length (ECL) value of 13.40, indicating multiple branching. In fact, the mass spectrum of the latter compound presented key fragments at m/z = 101 (100%), and at m/z = 171 (15%). These fragments clearly revealed methyl branching at positions 3 and 7. A literature comparison (8) indicated that the isolated compound was the isoprenoid fatty acid 3,7,11-trimethyldodecanoic (1), unprecedented in marine sponges (Scheme 1).

Three different methyl hexadecanoic acids were identified in *X. muta.* Two of these presented ECL values of 16.62 and 16.70, which definitely match the known values for iso and anteiso methyl hexadecanoic acids (9). When

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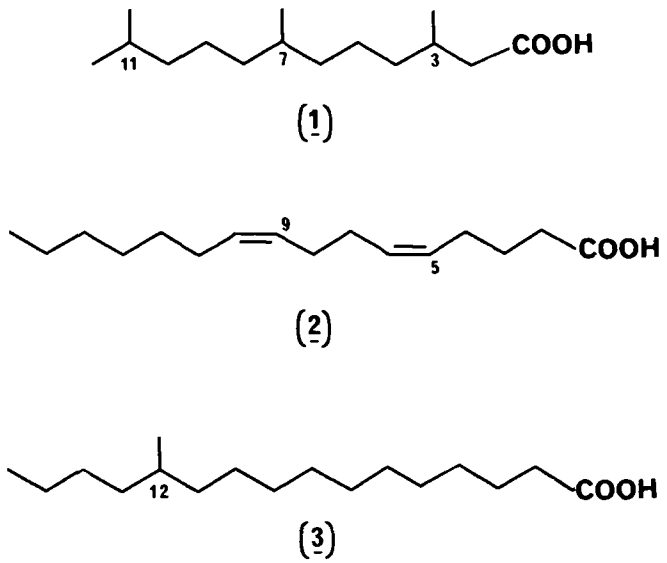
Abbreviations: ECL, equivalent chain length; PLC, preparative larger chromatography; GC-MS, gas chromatography-mass spectrometry.

TABLE 1

Identified Phospholipid Fatty Acids From *Xestospongia Muta*

Fatty acids	Abundance (%)
3,7,11-Trimethyldodecanoic (15:0)	3.3
Tetradecanoic (14:0)	2.4
12-Methyltetradecanoic (15:0)	9.4
Pentadecanoic (15:0)	1.4
14-Methylpentadecanoic (16:0)	1.3
5,9-Hexadecadienoic (16:2)	10.5
9-Hexadecenoic (16:1)	11.5
Hexadecanoic (16:0)	10.0
12-Methylhexadecanoic (17:0)	10.2
15-Methylhexadecanoic (17:0)	1.9
14-Methylhexadecanoic (17:0)	1.7
Heptadecanoic (17:0)	1.1
Octadecanoic (18:0)	4.9
16-Methyloctadecanoic (19:0)	7.7
Nonadecanoic (19:0)	1.2
Docosanoic (20:0)	12.5
Behenic (21:0)	1.6
5,9,19-Octacosatrienoic (28:3)	7.4



SCHEME 1

the corresponding pyrrolidies were prepared and their mass spectra taken, these compounds were definitely characterized as the known 15-methyl-hexadecanoic (iso) and 14-methyl-hexadecanoic (anteiso) acids. It is well-established in the mass spectrometry of branched pyrrolidides that if a peak of lower intensity than the analogous peak in a straight chain fatty acid pyrrolidide is observed, then this is indicative of methyl branching at that position (9). The third fatty acid methyl ester in the mixture presented a rather low ECL value of 16.40. This low value is characteristic of fatty acid methyl esters with methyl branching in the middle of the chain (10). Likely positions for the methyl group were 10, 11 and 12, because similar acids have been reported to occur in marine sponges (10). We decided to prepare the corresponding pyrrolidide to determine exactly the methyl group position. The pyrrolidide indicated the absence of a peak at m/z = 252 (carbon 12), while other fragments were present at intervals of 14 amu, that is, peaks at m/z = 224, 238, 266, 280, 294, 308, etc. Therefore, we concluded that the acid in question is 12-methylhexadecanoic (3) (Scheme 1), which has been isolated before from the marine sponge *Parasperella psila* (11).

From Table 1 we can also see that major acids in *Xestospongia muta* were palmitic (16:0), 9-hexadecenoic (16:1) and 5,9-hexadecadienoic (16:2). The latter acids accounted for more than 30% of the total mixture. For the characterization of the 5,9-hexadecadienoic acid (2), capillary GC-MS was used. The base peak in the mass spectrum of 5,9-hexadecadienoic methyl ester was observed at m/z = 81, a typical value for methyl esters with the Δ5,9-unsaturation. Upon catalytic hydrogenation (PtO_2), the hexadecadienoic acid methyl ester was converted to palmitic acid methyl ester, thus excluding the possibility of any branching. The double bonds in the molecule also were located by means of pyrrolidide derivatives. The typical peak at m/z = 180 was observed resulting from allylic cleavage (12). In fact, we isolated this acid some time ago from the sponge *Chondrilla nucula* that was identical to the one in this study (12). Pyrrolidides also provided an answer for locating the double bond in the 9-hexadecenoic acid isolated in this work.

A very long chain fatty acid methyl ester with molecular weight of m/z 432 also was obtained from *X. muta*. Hydrogenation of this methyl ester produced octacosanoic acid (28:0) as characterized by GC-MS. The fatty acid methyl ester of the original compound presented the typical base peak at m/z = 81, indicating Δ5,9 unsaturation. In accordance with previous findings by Litchfield and Marcantonio (5) in *X. halichondroides*, the most likely structure for our compound is that of the 5,9,19-octacosatrienoic acid (28:3). In contrast to Litchfield's results in *X. halichondroides*, we could not detect any 5,9-hexacosadienoic acid (26:2) in *X. muta*.

DISCUSSION

Last year, we carried out a brief survey of isoprenoid fatty acids from marine sponges (8) and concluded that only two isoprenoid fatty acids are found in marine sponges, i.e., the 4,8,12-trimethyltridecanoic acid and the 3,7,11,15-tetramethylhexadecanoic acid (phytanic). We also found that these isoprenoid fatty acids did not occur concomitantly in the same sponge. In this work, we report the isolation of still a third isoprenoid fatty acid not found previously in marine sponges, the 3,7,11-trimethyldodecanoic acid (1). This isprenoid fatty acid has been isolated before from a sediment of Eocene age, the Green River Shale, Colorado (13). Many natural products based on the isoprenoid skeleton could conceivably have given rise to isoprenoid fatty acid (1), but because it is related to phytanic acid, a common biosynthetic route should be operative. It has been established that the phytyl side chain of chlorophyll is the major precursor of phytanic acid (13). Therefore, it is very likely that the 3,7,11-trimethyldodecanoic acid isolated in this sponge could have been the product of oxidative degradation of

phytanic acid. Much work still lies ahead for the elucidation of the origin of these isoprenoid fatty acids in marine sponges.

Another interesting finding in *X. muta* is the isolation of the rare 5,9-hexadecadienoic acid (2), which we also have isolated before from the marine sponge *C. nucula* (12). In both cases, where we have been able to isolate the rare 5,9-hexadecadienoic acid (2), no 5,9-hexacosadienoic acid (26:2) has been found. On the other hand, in the many sponges that the "demospongic" acid 5,9-hexacosadienoic acid (26:2) has been identified, no 5,9-hexadecadienoic acid has ever been detected. This observation immediately brings interesting biosynthetic possibilities. Does the enzymatic system for oxidative desaturation in the sponges that contain the 5,9-hexadecadienoic acid operate at short chain lengths? To answer this question, biosynthetic experiments are in progress.

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REFERENCES

1. de Laubenfels, M.W. (1953) *Bull. Mar. Sci. Gulf and Carib. 2,* 511-557.
2. Hartman, W.D. (1973) *Discovery 9,* 13-26.
3. Li, L.N., Sjöstrand, U., and Djerassi, C. (1981) *J. Am. Chem. Soc. 103,* 115-119.
4. Schmitz, F.J., and Gopichand, Y. (1978) *Tetrahedron Lett. 39,* 3637-3640.
5. Litchfield, C., and Marcantonio, E.E. (1978) *Lipids 13,* 199-202.
6. Privett, O.S., Dougherty, K.A., Erdahl, W.L., and Stolywho, A. (1973) *J. Am. Oil Chem. Soc. 50,* 516-520.
7. Carreau, J.P., and Dubacq, J.P. (1978) *J. Chromatogr. 151,* 384-390.
8. Carballeira, N., Maldonado, L., and Porras, B. (1987) *Lipids 22,* 767-769.
9. Andersson, B.A. (1978) *Prog. Chem. Fats Other Lipids 16,* 279-308.
10. DasGupta, A., Ayanoglu, E., and Djerassi, C. (1984) *Lipids 19,* 768-776.
11. DasGupta, A., Ayanoglu, E., Wegmann-Szente, A., Tomer, K., and Djerassi, C. (1986) *Chem. Phys. Lipids 39,* 335-347.
12. Carballeira, N., and Maldonado, L. (1986) *Lipids 21,* 470-471.
13. MacLean, I., Eglinton, G., Douraghi-Zadeh, K., Ackman, A.G., and Hooper, S.N. (1968) *Nature 218,* 1019-1024.

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Milk Fat Globules: Fatty Acid Composition, Size and in vivo Regulation of Fat Liquidity

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Populations of large and small milk fat globules were isolated and analyzed to determine differences in fatty acid composition. Globule samples were obtained by centrifugation from milks of a herd and of individual animals produced under both pasture and barn feeding. Triacylglycerols of total globule lipids were prepared by thin layer chromatography and analyzed for fatty acid composition by gas chromatography. Using content of the acids in large globules as 100%, small globules contained fewer short-chain acids, −5.9%, less stearic acid, −22.7%, and more oleic acids, +4.6%, mean values for five trials. These differences are consistent with alternative use of short-chain acids or oleic acid converted from stearic acid to maintain liquidity at body temperature of milk fat globules and their precursors, intracellular lipid droplets. Stearyl-CoA desaturase (EC 1.14.99.5), which maintains fluidity of cellular endoplasmic reticulum membrane, is suggested to play a key role in regulating globule fat liquidity. Possible origins of differences between individual globules in fatty acid composition of their triacylglycerols are discussed.
Lipids 23, 685–689 (1988).

Whether there are differences in fatty acid composition between individual bovine milk fat globules is a question of both fundamental and practical importance. Such differences could imply heterogeneity in the cellular synthesis-assembly of milk fat globules. At the practical level, compositional variations suggest the possibility of fat globules with assorted properties to meet needs of various dairy products.

Milk fat globules are unique products of the lactating cell. Triacylglycerols synthesized in the rough endoplasmic reticulum (1–3) accumulate into droplets that are secreted from the cell by envelopment in plasma membrane and, to some extent, associated secretory vesicle membrane (4–6). For the bovine, these globules number about 1.5×10^{10} per ml of milk. They range in diameter from <1 μm to 20 μm with a volume surface average diameter of 3.4 μm. Globules <1 μm in diameter, while representing about 75%–80% of the total number, account for only a few percent of the lipid mass (7). On a dry basis, both bovine and human milk fat globules are about 1% protein (7,8) and 99% lipid (by difference). Of the lipid, 96% to 99% is triacylglycerol (7,9). Thus, milk fat globules are relatively pure particles of triacylglycerols.

The principal evidence that there are substantial differences in the fatty acid composition of individual globules of a milking from a single animal is based on refractive index (n^D) measurements by Walstra and Borggreve (10). They found that the spread in n^D among individual globules was 7×10^{-4}. Based on the relationship of n^D to melting properties of milk fat, these authors concluded that in average milk at least a fourth of the fat globules contain a hard fat and another fourth soft fat.

It is not yet possible to determine fatty acid composition of individual globules. However, size distribution curves for milk fat globules (11,12) and morphometric studies of intracellular globule origins (13,14) suggest possible compositional differences between populations of very small and larger globules. These populations can be prepared by centrifugal separation, which segregates the larger ones into cream and the smaller ones with the skim milk (15). We report here an investigation of fatty acid composition in triacylglycerols from such populations of globules under several conditions of feeding and managing lactating cows.

MATERIALS AND METHODS

Milk samples. Milk was obtained from Holstein cows at the Schaedtbek experimental farm of the Federal Dairy Research Center in Kiel, West Germany. Depending on the experiment, aliquots of complete milkings from single animals or of mixed herd milk from the farm bulk tank were used. Samples were obtained under conditions of pasture feeding, barn feeding and underfeeding. Barn feeding was composed of grass or wheat silage, supplemented by grain concentrate according to milk yield. Pasture feeding was supplemented by corn cob silage. Underfeeding was accomplished by giving the cow exclusively 8 kg wheat straw daily for one wk before the milk sample was taken. Samples (1 L) taken in the morning were immediately brought to the laboratory for further processing. Those from individual animals were uncooled; bulk tank samples were cooled to 2–4 C.

Separating large and small fat globules. After warming to 30 C and mixing the milk sample, 400 to 500 ml was centrifuged at $300 \times g$ for 15 min in 20-ml glass tubes plugged at the bottom end with rubber stoppers. Following centrifugation, the tubes were placed in ice water for about 20 min to harden the cream layers, after which the stoppers were removed and the skim milk decanted from the cream plug. The latter was used as the sample of larger globules. To obtain the smaller globules free of other lipid material (membranous vesicles, cell fragments), the skim milk was further centrifuged at $33,000 \times g$ and 4 C for one hr. The very thin cream layer obtained was carefully removed and suspended in water. This served as the sample of smaller globules and, on average, represented 5% of total globule lipid.

Determination of globule diameter. Samples of cream layers from both centrifugations were diluted with warm water and mixed with a 5% solution of gelatin in warm water, final temperature 30 C. An aliquot of this was transferred to the depression in a specially designed glass

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Abbreviations: The shorthand convention in the nomenclature for fatty acids of number: number representing carbon chain length and number of double bonds, respectively, is used throughout; br–, branched carbon chain.

microscope slide. A cover glass then was placed over the depression, thereby creating a 0.01-mm thick film containing the globules. The slide was cooled in a refrigerator (5 C) to solidify the gelatin and, after readjustment to room temperature, was viewed with a camera-equipped Leitz Orthomat microscope. Regions of good globule distribution (free of clumps) were photographed. Positive enlargements, total magnification 1790×, were used to measure fat globule diameters. These were determined with the aid of an electronic pencil and tablet to trace 2000 to 3000 globule circumferences per preparation, and a microcomputer to process the data (16). The diameters were calculated as d_n, the number average diameter. This method of assaying globule size, while not adequate in accounting for the huge numbers of very small globules in milk (11), is sufficient to show significant differences in the globule sizes between cream and skim milk.

Preparation and fatty acid analysis of lipids. Whole milk samples and dispersed cream layers were solvent extracted to obtain total lipids by the Röse-Gottlieb procedure (17) as modified by Walstra and de Graaf (18). Polar (membrane) lipids represent a relatively larger component of small as compared with large globules. To obtain compositional data more representative of the globule core, we isolated triacylglycerols of lipid samples by thin layer chromatography. Each sample (30 to 40 mg) was applied the length of one dimension on a precoated 0.25 mm, 20 × 20 cm silica gel thin layer chromatography plate (No. 5721, Merck, Darmstadt, West Germany). Separation of lipids was achieved with petroleum ether/ethyl ether/acetic acid (75:25:1, v/v/v). The band of triacylglycerols (R_f 0.7 to 0.8, detected by covering all but 1 cm at the running edge of the plate and exposing it to iodine vapor, was scraped off with a razor blade and extracted from the silica gel with diethyl ether.

Triacylglycerols were weighed, made to a 5% solution in redistilled heptane and transesterified with sodium methoxide in methanol (19). The resulting methyl esters were analyzed in a Packard-Becker Model 438 gas chromatograph (Delft, Netherlands) with a Spectra Physics 4100 computing integrator under the following conditions: 25 m × 0.23 mm (i.d.) fused silica capillary column coated with SP-1000 (Supelco, Bellefonte, PA); injector, flame ionization detector and oven program temperatures of 220, 260 and 50–235 C, respectively; sample (1 μl) injection with a 1:100 split, column flow of 1 ml/min and additional detector gas flow (auxiliary gas) of 29 ml/min (both N_2). A precisely weighed test mixture of fatty acid methyl esters closely approximating an average milk fat was used to calculate correction factors for peak areas on sample chromatograms. The results were expressed as wt. % of each fatty acid (as acid) in relation to the total fatty acids (as acids). Data in the tables are averages of two gas chromatographic runs for each sample. This methodology for quantitation of fatty acids was developed by scientists of the International Dairy Federation and the European Economic Community for analysis of fats and oils, including milk fat.

RESULTS

Fatty acid composition of total lipid samples from milks of individual animals and of the herd under pasture and barn feeding conditions are presented in Table 1. With

TABLE 1

Fatty Acid Composition of Total Lipids from Milks Produced under Various Conditions

	Wt. %					
Fatty acids	Trial 1 Herd Pasture fed	Trial 2 Individual Pasture fed	Trial 3 Herd Barn fed	Trial 4 Individual Barn fed	Trial 5 Individual Barn fed	Trial 6 Individual Underfed
4:0	3.72	3.83	3.77	3.94	3.79	3.08
6:0	2.15	2.39	2.48	2.61	2.41	1.47
8:0	1.25	1.43	1.51	1.56	1.44	0.68
10:0	2.64	3.01	3.63	3.56	3.49	1.26
10:1	0.34	0.48	0.34	0.42	0.40	0.12
12:0	3.43	3.66	4.80	4.81	4.61	1.31
12:1	0.11	0.15	0.11	0.13	0.15	0.03
13:0 br	0.17	0.22	0.15	0.18	0.19	0.06
13:0	0.09	0.07	0.18	0.14	0.14	0.04
14:0 br	0.14	0.12	0.10	0.08	0.09	0.05
14:0	10.41	11.01	12.56	11.92	12.76	6.17
14:1	1.18	1.62	1.14	1.09	1.64	1.00
15:0 br	0.96	0.83	0.69	0.70	0.60	0.36
15:0	1.12	0.96	1.35	1.24	1.05	0.54
16:0 br	0.25	0.21	0.20	0.18	0.21	0.22
16:0	26.63	29.73	35.40	36.42	43.74	27.71
16:1	2.17	2.23	2.14	1.98	2.62	4.08
17:0 br	1.10	1.02	0.96	1.00	0.89	1.00
17:0	0.78	0.76	0.62	0.52	0.50	0.82
17:1	0.37	0.31	0.31	0.26	0.24	0.76
18:0	10.02	9.64	6.48	6.49	5.04	7.85
18:1	25.23	21.71	16.90	16.98	11.26	37.47
18:2	2.20	1.72	2.08	2.27	1.46	2.19
18:2[a]	1.34	0.77	0.27	0.34	0.17	0.46
18:3	0.84	0.82	0.36	0.31	0.22	0.46
20:0	0.29	0.24	0.15	0.10	0.09	0.12
20:1	0.16	0.14	0.24	0.10	0.11	0.17
>20:1	0.63	0.69	0.60	0.33	0.30	0.24

[a]Conjugated double bond.

the exception of data from the underfed animal, the principal variations related to elevated 18:0 and 18:1 and lower 16:0 in the pasture feeding as compared with barn feeding trials. This is a characteristic difference that can be attributed to prevalence of 18: acids in pasture lipid (20–22). In general, not only 16:0 was increased under barn feeding, but all the major short and medium chain fatty acids. This effect was least with 4:0. With underfeeding, 4:0 and 16:0 were depressed. This was compensated mainly by increased 18:1. Similar effects of underfeeding have been observed by us and others (22,23). The following fatty acids, occurring rather consistently below 0.1%, have been omitted from the data of Table 1: 5:0, 7:0, 9:0, 11:0, 15:1, 18:0 br, 19:0.

Data illustrating the differences in fatty acid composition of triacylglycerols of whole milk, large globules of cream, and small globules from skim milk are presented in Table 2. For the sake of space, complete data for only herd milk under barn feeding are given as an example. The principal differences are shown by the small globules. Data for whole milk and the cream (larger globules) are virtually identical. This would be expected in that the cream sample accounts for 95% or more of the milk triacylglycerols. The close agreement of data for the whole milk and its cream also reveals the precision of the analytical method. Data variation between larger globules and smaller globules (given in the right hand columns of the table) show slightly less 4:0 to 10:0, slightly more 12:0 and 14:0, virtually no change in 16:0, less 18:0 and greater 18:1 for the small globules. The differences in fatty acid composition of triacylglycerols of larger vs smaller globules for six trials are summarized in Table 3. Mean arithmetic globule diameters for the samples are also included. These data establish that, in all of the normal feeding trials, 1–5, triacylglycerols of the smaller globules were associated with reduced 4:0 to 10:0 and 18:0 and increased 18:1 in comparison with results for the larger globules. Each of the short chain acids was lower in the

TABLE 2

A Comparison of Fatty Acid Composition of Triacylglycerols from Herd Milk and from Globules of Its Cream and Skim Milk[a]

Fatty acids	Whole milk wt. %	Cream wt. %	Skim milk wt. %	Differences Skim vs cream	
				Actual wt. %	Relative wt. %[b]
4:0	3.73	3.80	3.63	−0.17	−4.5
5:0	0.05	0.05	0.05		
6:0	2.46	2.50	2.41	−0.09	−3.6
7:0	0.05	0.05	0.06		
8:0	1.50	1.54	1.45	−0.09	−5.8
9:0	0.07	0.07	0.08		
10:0	3.62	3.67	3.54	−0.13	−3.5
10:1	0.33	0.34	0.34		
11:0	0.14	0.14	0.14		
12:0	4.80	4.82	4.88	+0.06	+1.2
12:1	0.12	0.11	0.13		
13:0 br	0.15	0.15	0.18		
13:0	0.23	0.21	0.25		
14:0 br	0.09	0.11	0.09		
14:0	12.45	12.46	12.72	+0.26	+2.1
14:1	1.14	1.14	1.23		
15:0 br	0.68	0.69	0.72		
15:0	1.33	1.34	1.43		
15:1	0.06	0.06	0.07		
16:0 br	0.20	0.20	0.21		
16:0	35.08	35.11	35.02	−0.09	−0.3
16:1	2.17	2.13	2.24		
17:0 br	0.97	0.07	0.99		
17:0	0.62	0.62	0.62		
17:1	0.34	0.34	0.33		
18:0 br	0.06	0.09	0.05		
18:0	6.56	6.56	5.78	−0.78	−11.9
18:1	16.97	16.98	17.51	+0.53	+3.1
18:2	2.06	2.03	2.10		
18:2[c]	0.25	0.26	0.24		
19:0	0.06	0.06	0.06		
18:3	0.35	0.35	0.37		
20:0	0.17	0.17	0.14		
20:1	0.27	0.25	0.28		
>20:1	0.90	0.66	0.69		
Total	100.0	100.0	100.0		

[a]Trial 3 under barn (winter) feeding conditions.

[b]Derived on the basis of the actual percentage in cream as 100%.

[c]Conjugated double bond.

TABLE 3

Differences between Fat Globules of Creams and Corresponding Skim Milks in Triacylglycerol Fatty Acid Composition for Milks Produced under Various Conditions[a]

	Trial 1 Herd Pasture fed	Trial 2 Individual Pasture fed	Trial 3 Herd Barn fed	Trial 4 Individual Barn fed	Trial 5 Individual Barn fed	Trial 6 Individual Underfed
Globule size[b]						
Cream	2.81	2.93	3.17	n.d.	2.76	3.33
Skim milk	1.41	1.02	1.77	n.d.	1.50	1.35
Fatty acids			wt. %			
4:0-10:0	−0.21	−0.48	−0.48	−0.79	−1.38	+0.12
12:0	0	−0.02	+0.06	−0.10	−0.43	+0.19
14:0	+0.01	+0.02	+0.26	0	−0.41	+0.59
16:0	−1.07	+0.95	−0.09	+0.78	+0.10	+0.99
18:0	−0.98	−0.98	−0.78	−0.89	−0.60	−0.48
18:1	+0.73	+0.06	+0.53	+0.97	+1.24	−1.22
18:2 + :3	+0.66	+0.19	+0.07	−0.15	+0.22	−0.02

[a]Differences are wt. % composition of triacylglycerol fatty acids from skim milk less those from cream.
[b]Arithmetic mean of globule size diameters in μm (d_n).

smaller globules for all five trials, the mean differences in actual % being: 4:0 −0.21, 6:0 −0.14, 8:0 −0.09, 10:0 −0.20. The other acids in the table, i.e., 12:0, 14:0, 16:0, 18:2 and 18:3, showed no consistent trend. In terms of relative differences, using content of the acids in larger globules as 100%, the mean values were −5.9, −11.7 and +4.6% for short chain, stearic and oleic acids, respectively. Considering the normal fatty acid composition of milk triacylglycerols (Table 1), this reduction in stearic acid, in terms of mass, is rather precisely equivalent to the gain in oleic acid.

Differences in fatty acids for large and small fat globules of the underfed animal (Trial 6, Table 3) bear little or no relationship to data for the other five trials. Because many normal synthetic and secretory pathways within the lactating cell may be perturbed under such stress, it is difficult to rationalize these data at this time. However, in additional trials (not shown) the trends of data for this trial, as well as those in the other five trials, were reproducible.

DISCUSSION

Profound differences in fatty acid composition between large and small fat globules were not found in this study (Table 3). However, the small differences, consistently detected, seem important for a number of reasons. It is notable that the fatty acids involved are crucial to the liquidity of milk fat. It is a liquid at body temperature (24), presumably of necessity for its synthesis and secretion by the lactating cell, as well as for efficient utilization by the offspring. The principal means of assuring this liquidity is the incorporation of oleic acid (18:1, mp 14 C), which is produced from stearic acid (18:0, mp 69 C) in bovine mammary tissue (25-27), and of short chain fatty acids (4:0-10:0, mp −8 to +31 C) into triacylglycerols destined for milk fat globules. Thus, it is reasonable to interpret increases in 18:1 with corresponding decreases in 18:0 as a response to meet liquidity requirements of cytoplasmic fat droplets due to lesser availability of 4:0 to 10:0. Because these small differences are seen as a net effect in a substantial fraction of the total globule population, much larger differences may occur in some individual globules. This is in keeping with the significant differences observed in refractive indices between globules of equal size by Walstra and Borggreve (10). However, in distinction to their interpretation that this means half of the total globules have either relatively solid or liquid fat, this could mean liquidity being maintained by greater incorporation of oleic acid in some globules, thus increasing refractive index, or by greater incorporation of short chain acids in other globules, which would lower the index. Refractive indices (n_D) for the various acids and milk fat are: 4:0, 1.399^{20}; 6:0, 1.416^{20}; 8:0, 1.428^{20}; 10:0, 1.429^{40}; 18:0, 1.430^{80}; 18:1, 1.463^{18} (28); and milk fat (butterfat), $1.4530-1.4555^{40}$ (29).

The conversion of stearic acid to oleic acid in lactating bovine tissue is accomplished by the microsomal enzyme, stearyl-CoA desaturase (EC 1.14.99.5) (25-27). Data reviewed by Thompson (30) show that such desaturases are stimulated by increase in membrane rigidity, a condition that is controlled primarily by membrane lipid composition as well as by temperature. Because newly produced triacylglycerols and their precursor diglycerides would be expected to influence the fluidity-rigidity of membranes accomplishing their synthesis, it is evident that the membrane fluidity-desaturase activity relationship could provide a basic control mechanism for maintaining liquidity of accumulating fat droplets.

As to the origin of compositional differences between large and small globules, one explanation may lie in the mechanism by which intracellular milk lipid droplets grow in size. There is growing evidence that this occurs by a process of small droplet fusion to produce large droplets (13,14,31,32). In detailed studies of the ultrastructure and biochemistry of lipid droplets in the lactating cell (3,31,32), microlipid droplets with diameters <0.5 μm originating in the endoplasmic reticulum were isolated

and characterized. These studies provide evidence that the microlipid droplets may fuse to form larger cytoplasmic droplets or be secreted as such. One interpretation of our findings is that not all of the intracellular microlipid droplets fuse with equal facility; in particular, those containing less short chain fatty acids may be more resistant and tend to be secreted directly or with few fusions. However, observed significant differences in refractive indices of medium-sized milk fat globules (3.5 to 6.5 μm) (10) suggest that there must be differences in fatty acid composition of larger intracellular lipid droplets as well.

Another factor that may cause differences between globules in fatty acid composition is spatial asymmetry in supply and incorporation of the acids. It has been suggested (33) that the basal region of the lactating cell is enriched in long chain fatty acids from the circulation, whereas the mid- to upper-region may be the primary source of short chain acids, these being synthesized de novo within mammary tissue (34). It also is true that if exchange of short chain fatty acids for oleate were occurring at the surface of intracellular lipid droplets, more exchange would take place per unit mass of small droplets because of their larger surface to volume relationship. Other asymmetries may concern varying supply of precursors for milk lipid synthesis at the gland and whole body levels.

In light of observations to date on globule size and composition together with findings on lipid droplets of the lactating cell, it is possible to explain the three (overlapping) size subpopulations of milk fat globules noted by Walstra (11) and Rüegg and Blanc (12). Small globules result from secretion of the microlipid droplets of Deeney et al. (32), which have undergone no or limited fusions, the intermediate-sized globules arise from cytoplasmic droplets resulting from many fusions of microdroplets and, as suggested by Walstra (11), the larger globules, ranging sometimes from 20 μm in diameter, represent post-secretion fusion of globules, because in some cases they tend to exceed dimensions of the cell.

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REFERENCES

1. Stein, O., and Stein, Y. (1967) *J. Cell Biol. 34,* 251-263.
2. Kinsella, J.E. (1972) *Lipids 7,* 165-170.
3. Valivullah, H.M., Dylewski, D.P., and Keenan, T.W. (1986) *Int. J. Biochem. 18,* 799-806.
4. Patton, S., and Keenan, T.W. (1975) *Biochim. Biophys. Acta 415,* 273-309.
5. McPherson, A.V., and Kitchen, B.J. (1983) *J. Dairy Res. 50,* 107-133.
6. Keenan, T.W., Dylewski, D.P., Woodford, T.A., and Ford, R.H. (1983) in *Developments in Dairy Chemistry—2* (Fox, P.F., ed.) pp. 83-118, Applied Science Publishers, Essex, England.
7. Walstra, P., and Jenness, R. (1984) *Dairy Chemistry and Physics,* pp. 58-91, 254-255, John Wiley and Sons, New York.
8. Patton, S., and Huston, G.E. (1986) *Lipids 21,* 170-174.
9. Lammi-Keefe, C.J., and Jensen, R.G. (1984) *J. Pediatr. Gastroenterol. Nutr. 3,* 172-198.
10. Walstra, P., and Borggreve, G.J. (1966) *Neth. Milk Dairy J. 20,* 140-143.
11. Walstra, P. (1969) *Neth. Milk Dairy J. 23,* 99-110.
12. Rüegg, M., and Blanc, B. (1981) *Biochim. Biophys. Acta 666,* 7-14.
13. Stemberger, B.H., and Patton, S. (1981) *J. Dairy Sci. 64,* 422-426.
14. Stemberger, B.H., Walsh, R.M., and Patton, S. (1984) *Cell Tissue Res. 236,* 471-475.
15. Mulder, H., and Walstra, P. (1974) *The Milk Fat Globule,* pp. 54-61, 159, Pudoc, Wageningen, The Netherlands.
16. Donnelly, W.J., McNeill, G.P., Buchheim, W., and McGann, T.C.A. (1984) *Biochim. Biophys. Acta 789,* 136-143.
17. Horowitz, W., ed. (1975) in *Official Methods of Analysis of the AOAC,* p. 258, Association of Official Analytical Chemists, Washington, D.C.
18. Walstra, P., and de Graaf, J.J. (1962) *Neth. Milk Dairy J. 16,* 283-287.
19. Christopherson, S.W., and Glass, R.L. (1969) *J. Dairy Sci. 52,* 1289-1290.
20. Patton, S., McCarthy, R.D., Evans, L., and Lynn, T.R. (1960) *J. Dairy Sci. 43,* 1187-1195.
21. Shorland, F.B., Weenink, R.O., Johns, A.T., and McDonald, I.R.C. (1957) *Biochem. J. 67,* 328-333.
22. Precht, D., Frede, E., Hagemeister, H., and Timmen, H. (1985) *Fette Seifen Anstrichm. 87,* 117-126.
23. Hilditch, T.P., and Williams, P.N. (1964) in *The Chemical Constitution of Natural Fats,* 4th edn., pp. 153-154, Chapman and Hall, London.
24. Sherbon, J.W., and Dolby, R.M. (1972) *J. Dairy Res. 39,* 319-326.
25. Lauryssens, M., Verbeke, R., and Peters, G. (1961) *J. Lipid Res. 2,* 383-388.
26. Annison, E.F., Linzell, J.L., Fazakerely, S., and Nichols, B.W. (1967) *Biochem. J. 102,* 637-647.
27. Kinsella, J.E. (1972) *Lipids 7,* 349-355.
28. Windholz, M., ed. (1983) in *The Merck Index,* 10th edn., pp. 1-1463, Merck and Co., Rahway, NJ.
29. Schulz, M.E., and Voss, E. (1965) in *Das Grosse Molkerei-Lexikon,* 4th edn., p. 977, Volkswirtschaftlicher Verlag G.m.b.H., Kempten, Allgäu, West Germany.
30. Thompson, G.A. (1980) in *The Regulation of Membrane Lipid Metabolism,* pp. 171-196, CRC Press Inc., Boca Raton, FL.
31. Dylewski, D.P., Dapper, C.H., Valivullah, H.M., Deeney, J.T., and Keenan, T.W. (1984) *Eur. J. Cell Biol. 35,* 99-111.
32. Deeney, J.T., Valivullah, H.M., Dapper, C.H., Dylewski, D.P., and Keenan, T.W. (1985) *Eur. J. Cell Biol. 38,* 16-26.
33. Patton, S., and McCarthy, R.D. (1963) *J. Dairy Sci. 46,* 916-921.
34. McCarthy, S., and Smith, G.H. (1972) *Biochim. Biophys. Acta 260,* 185-196.

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7-Methyl-8-hexadecenoic Acid: A Novel Fatty Acid from the Marine Sponge *Desmapsama anchorata*

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The novel fatty acid 7-methyl-8-hexadecenoic (*1*) was identified in the marine sponge *Desmapsama anchorata*. Other interesting fatty acids identified were 14-methyl-8-hexadecenoic (*2*), better known through its methyl ester as one of the components of the sex attractant of the female dermestid beetle, and the saturated fatty acid 3-methylheptadecanoic (*3*), known to possess larvicidal activity. The main phospholipid fatty acids encountered in *D. anchorata* were palmitic (16:0), behenic (22:0) and 5,9-hexacosadienoic acid (26:2), which together accounted for 50% of the total phospholipid fatty acid mixture.
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Sponges have been the source of many interesting phospholipid fatty acids possessing unusual unsaturation and methyl branching alternatives not found in terrestrial counterparts (1). The finding by Litchfield et al. of marine phospholipid fatty acids (C26–C30) with an unusual Δ5,9 unsaturation (2) as well as our recent report (3) of the isolation of the rare 5,9-hexadecadienoic acid from the marine sponge *Chondrilla nucula* are interesting. A good example of methyl branching in marine sponges can be found in our recent report of isoprenoid fatty acids from common Caribbean sponges (4) as well as in the original work of Walkup et al. (5). In this paper, however, we would like to report the isolation of several unusual phospholipid fatty acids that combine, in the same structure, unusual unsaturation with odd methyl branching. The phospholipid fatty acids that we report here are the novel 7-methyl-8-hexadecenoic (1), the important 14-methyl-8-hexadecenoic (2), and the rare 3-methylheptadecanoic (3), which were isolated from the Caribbean sponge *Desmapsama anchorata* (Scheme 1). To the best of our knowledge, 7-methyl-8-hexadecenoic is new in nature. The corresponding saturated branched analogue 7-methylhexadecanoic acid has been isolated from sperm whale oil (6) and the linear unsaturated 8-hexadecenoic acid has been detected in several biota, including green and blue-green algae (7). It is important to mention that 14-methyl-8-hexadecenoic acid methyl ester, together with 14-methyl-8-hexadecen-1-ol, have been reported to be the active constituents of the sex attractant of the female dermestid beetle *Trogoderma inclusum* and the khapra beetle *Trogoderma granarium*. These beetles are very serious pests in stored cereal products in hot, dry parts of the world, and they consume a wide variety of products such as grain, cereals and dried milk (8). Here we report the results of our investigation.

EXPERIMENTAL PROCEDURES

D. anchorata was collected January 21, 1987, at the depth of 50 feet near Cayo Enrique, Puerto Rico. The sponge was washed in sea water, carefully cleaned of all non-sponge debris and cut into small pieces. Immediate extraction with chloroform/methanol (1:1, v/v) yielded the total lipids. The neutral lipids, glycolipids and phospholipids were separated by column chromatography on silica gel, grade 62 (60–200 mesh) using the procedure of Privett et al. (9). The fatty acyl components of the phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic hydrogen chloride (10) followed by purification on column chromatography eluting with hexane/ether (9:1, v/v). The resulting methyl esters were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Hewlett-Packard 5995 A gas chromatograph-mass spectrometer equipped with a 30 × 0.32 mm fused silica column coated with SE-54. For the location of double bonds, N-acylpyrrolidide derivatives were prepared by direct treatment of the methyl esters with 2 ml pyrrolidine and catalytic amounts of acetic acid in a capped vial (3 days at 100 C) followed by ethereal extraction from the acidified solution and purification by column chromatography on silica gel. Hydrogenations were carried out in 5 ml of absolute methanol and catalytic amounts of platinum oxide (PtO_2). Mass spectral data of key fatty acids for this discussion are presented below.

7-Methyl-8-hexadecenoic acid methyl ester. MS m/z (rel. int.) 282 (M^+, 6), 251 (4), 250 (3), 168 (10), 167 (18), 151 (13), 138 (62), 137 (12), 125 (13), 123 (22), 115 (32), 114 (11), 111 (17), 110 (27), 109 (24), 97 (45), 95 (39), 93 (15), 91 (10), 87 (12), 85 (10), 83 (45), 81 (49), 79 (18), 74 (19), 71 (19), 70 (16), 69 (61), 67 (47), 55 (100).

7-Methyl hexadecanoic acid methyl ester. MS m/z (rel. int.) 284 (M^+, 5), 235 (2), 234 (2), 185 (5), 158 (3), 157 (27), 135 (4), 130 (4), 130 (4), 129 (13), 126 (3), 125 (12), 123 (2), 111 (4), 101 (8), 97 (22), 87 (71), 83 (15), 75 (23), 74 (100), 71 (11), 69 (22), 57 (29), 55 (47).

7-Methyl-8-hexadecenoic acid pyrrolidide. MS m/z (rel. int.) 321 (M^+, 6), 236 (1), 222 (2), 208 (5), 194 (2), 182 (1), 168 (3), 154 (6), 140 (5), 127 (11), 126 (36), 113 (100), 98 (23), 95 (4), 85 (10), 72 (28), 71 (17), 70 (40), 69 (18), 67 (13), 56 (17), 55 (56).

3-Methylheptadecanoic acid methyl ester. MS m/z (rel. int.) 298 (M^+, 7), 171 (13), 143 (17), 129 (8), 111 (14), 101 (84), 97 (21), 96 (13), 95 (16), 87 (35), 83 (26), 75 (42), 74 (100), 69 (61), 57 (34), 55 (58).

14-Methyl-8-hexadecenoic acid methyl ester. MS m/z (rel. int.) 282 (M^+, 6), 252 (I2), 251 (4), 250 (4), 221 (3), 168 (11), 167 (18), 155 (6), 152 (5), 151 (12), 139 (10), 138 (58), 137 (11), 125 (11), 123 (20), 115 (28), 114 (10), 111 (16), 110 (27), 109 (23), 97 (43), 96 (19), 95 (39), 93 (10), 87 (18), 85 (11), 84 (18), 83 (44), 81 (43), 74 (32), 69 (70), 68 (25), 67 (43), 55 (100).

14-Methylhexadecanoic acid methyl ester. MS m/z (rel. int.) 284 (M^+, 10), 255 (4), 253 (2), 242 (3), 241 (9), 227 (5), 213 (5), 199 (9), 185 (6), 143 (16), 129 (7), 125 (5), 111 (6), 101 (5), 97 (15), 87 (61), 75 (23), 74 (100), 69 (19), 57 (26), 55 (44).

14-Methyl-8-hexadecenoic acid pyrrolidide. MS m/z (rel.

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; ECL, equivalent chain length; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high pressure liquid chromatography; TLC, thin layer chromatography.

int.) 321 (M^+, 2), 208 (2), 194 (1), 182 (2), 168 (2), 154 (2), 140 (2), 127 (7), 126 (22), 113 (100), 98 (15), 85 (7), 71 (17), 70 (28), 69 (24), 57 (19), 55 (37).

RESULTS

Our results are presented in Table 1. As can be seen from the table, the main phospholipid fatty acids encountered in *D. anchorata* were palmitic (16:0), behenic (22:0) and 5,9-hexacosadienoic acids (26:2). The latter acids are typical acids from marine sponges and, in this case, they accounted for 50% of the total mixture. It is interesting to point out here that in most of the Caribbean sponges that we have analyzed, behenic (22:0) has been a predominant acid in the mixtures. The 5,9-hexacosadienoic acid (26:2), typical of the Demospongiae, was characterized by means of GC-MS, because fatty acid methyl esters possessing the 5,9-unsaturation reveal a base peak at m/z = 81, and the corresponding pyrrolidide derivatives a key peak at m/z = 180 resulting from allylic cleavage (11). Another interesting fatty acid possessing the 5,9-unsaturation was encountered in *D. anchorata*, namely the 5,9-octacosadienoic acid (28:2), which accounted for 0.9% of the total fatty acid mixture. Its characterization also was performed by means of GC-MS, affording the characteristic fragmentation patterns already discussed above. The phospholipid mixture was analyzed by means of thin layer chromatography (TLC) and shown to consist mainly of phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

Three rather unusual fatty acids for marine sponges were encountered in *D. anchorata*. The most interesting fatty acid methyl ester presented an equivalent chain length (ECL) value of 16.28 and a molecular weight of MW = 282. Upon catalytic hydrogenation, the latter fatty acid methyl ester was converted to a saturated ester (MW = 284) with an ECL value of 16.45 in a SE-54 capillary column. This result confirms the presence of methyl branching, but excludes any of the common iso or anteiso C17 fatty acid methyl esters, because these acids normally present ECL values between 16.60 and 16.69 (12). Moreover, the 16.45 value suggested that the hydrogenated product could be the 7-methylhexadecanoic acid methyl ester, because it has been reported (6) for the latter fatty acid methyl ester an ECL value of 16.4. To confirm what the ECL value was suggesting to us, we carefully looked at the mass spectrum of the hydrogenated product and found a most revealing fragmentation peak at m/z = 157, not significant in the mass spectra of the iso and anteiso C17 series, with a considerable abundance of 27%. The latter fragmentation peak clearly shows methyl branching at C-7, because this fragment corresponds to a favorable fragmentation at the branching between carbons 7 and 8, resulting in the stabilized $C_9H_{17}O_2^+$ cation. Therefore, this mass spectral data confirms C7 methyl substitution but, as definite proof for methyl branching and unsaturation, we prepared the corresponding pyrrolidide derivative of this acid and submitted the product to electron impact mass spectrometry. The results were very revealing, and they are shown in Figure 1, spectrum A. First, double bond unsaturation between carbons 8 and 9 was recognized due to a separation of 12 amu between fragments at m/z = 182 and 194. It is known from the mass spectra of fatty acid pyrrolidides that if an interval of 12 amu, instead of the regular 14, is observed between the most intense peaks of clusters of fragments containing n and n-1 carbon atoms in the acid moiety, a double bond occurred between carbon n and n+1 in the molecule (13). In our case, an interval of 12 amu was encountered between carbons 8 and 7, indicating unsaturation between carbons 8 and 9. Second, a peak of low intensity was observed at exactly m/z = 182, flanked by neighboring peaks higher than usual (Fig. 1), indicating C7 methyl branching. It is well-established in the mass spectrometry of branched pyrrolidides that, if a peak of lower intensity than the

TABLE 1

Identified Phospholipid Fatty Acids from *Desmapsama anchorata*

Fatty acids	Abundance (%)
Tetradecanoic (14:0)	1.2
Pentadecanoic (15:0)	0.3
14-Methylpentadecanoic (16:0)	0.5
Hexadecanoic (16:0)	11.2
7-Methyl-8-hexadecenoic (17:1)	4.3
14-Methyl-8-hexadecenoic (17:1)	8.4
Heptadecanoic (17:0)	0.4
3-Methylheptadecanoic (18:0)	0.9
11-Octadecenoic (18:1)	5.7
Octadecanoic (18:0)	3.9
Nonadecanoic (19:0)	1.7
Methyldocosanoic (20:0)	0.8
Docosanoic (20:0)	4.9
Heneicosanoic (21:0)	2.1
Behenic (22:0)	20.1
Tricosanoic (23:0)	1.2
Tetracosanoic (24:0)	11.9
Pentacosanoic (25:0)	1.7
5,9-Hexacosadienoic (26:2)	17.8
5,9-Octacosadienoic (28:2)	0.9

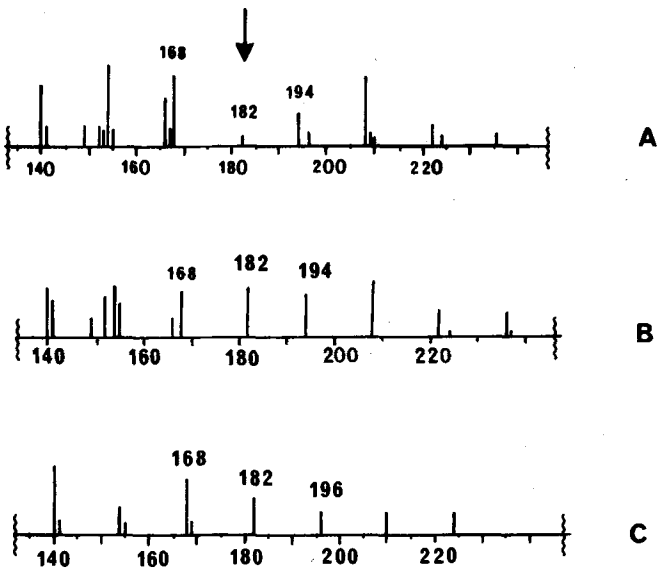
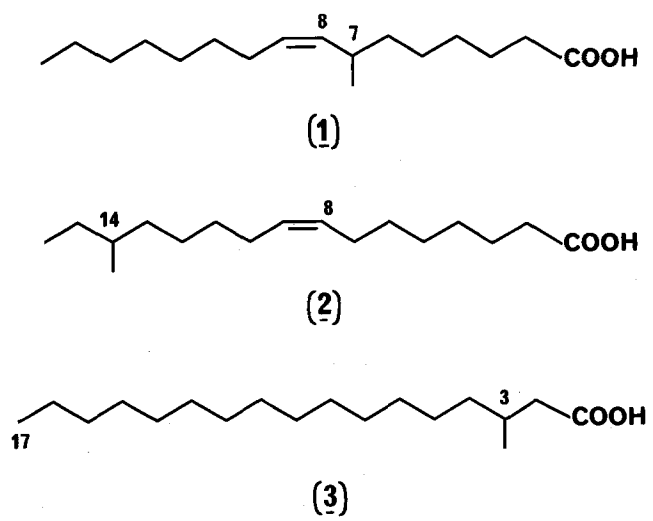


FIG. 1. Partial mass spectra of 7-methyl-8-hexadecenoic acid pyrrolidide (A), 14-methyl-8-hexadecenoic acid pyrrolidide (B) and behenic acid pyrrolidide (C). Spectra are expanded three times.

analogous peak in a straight-chain fatty acid pyrrolidide is observed, then this is indicative of a branched methyl at that position. From all of the above experimental data, we concluded that the acid in question is the unprecedented 7-methyl-8-hexadecenoic acid (1), which, to the best of our knowledge, has not been reported to occur in nature (Scheme 1).

Our second interesting fatty acid methyl ester occurred in the mixture at an abundance of 8%. The mass spectrum of this fatty acid methyl ester also presented a molecular peak at m/z = 282. Upon catalytic hydrogenation, this fatty acid methyl ester was converted to its saturated analogue (MW = 284), which presented an ECL value of 16.74, almost the same as that exhibited by an anteiso C17 fatty acid methyl ester. In fact, when coinjected in gas chromatography (GC) with an authentic sample of the anteiso C17 methyl ester obtained from other sponges that we have analyzed in our laboratory, the hydrogenated product proved to be identical. The position of the only double bond in the parent molecule was determined by preparing the corresponding pyrrolidide. The latter clearly presented an interval of 12 amu between m/z = 182 and m/z = 194 (Fig. 1, spectrum B), indicating also unsaturation between carbons 8 and 9. For comparison, we have included in Figure 1, spectrum C, the pyrrolidide derivative of the saturated linear behenic acid, where a difference of 14 amu is clearly seen between m/z = 182 and m/z = 196. From our mass spectral data and GC retention times, we concluded that we have isolated the known 14-methyl-8-hexadecenoic acid (2), most commonly known as its methyl ester as one of the components of the sex attractant of the female dermestid beetle *Trogoderma inclusum!*

Our third unusual fatty acid methyl ester presented an ECL value of 17.55, a rather low value. Mass spectral analysis revealed a molecular weight of 298, suggesting 18 carbons in the molecule and the base peak at m/z = 74 confirmed it to be a fatty acid methyl ester. This compound was inert upon catalytic hydrogenation, confirming branching and no unsaturation. The mass spectrum of the fatty acid methyl ester was very revealing, especially a peak at m/z = 101, not found in iso and anteiso fatty acids, with an impressive abundance of 84%. This clearly indicates methyl branching at carbon 3 due to a favorable cleavage between carbons 3 and 4. Because only one methyl branching can be explained from the observed ECL value, the original compound in question is the rare 3-methylheptadecanoic (3), which was observed in 0.9% abundance (Scheme 1).



SCHEME 1

Because sterols are known to coexist with phospholipids in cell membranes, we decided to take a look at the sterols from *D. anchorata* in detail to see if unusual methyl branching also was to be found in the sterol mixture. With the use of high pressure liquid chromatography (HPLC), 400-MHz ^{1}H-NMR, MS, and comparison with authentic samples, the following sterols were isolated and characterized from *D. anchorata.* Desmosterol (1.8% abundance of the total sterol mixture), cholesterol (41%), sitosterol (13%), stigmasterol (4.7%), 24-methylcholesterol (5.4%), 24(S)-methylcholesta-5,22-dienol (15.1%), 24(R)-methylcholesta-5,22-dienol (4.8%), 24-methylenecholesterol (4.6%), and cholesta-5,22-dienol (9.6%). Therefore, *D. anchorata* presented a common sponge sterol composition (14). However, we should mention in this context that when doing the analyses, we isolated several times reasonable amounts of a compound, which seems to be common in *D. anchorata,* presenting a molecular weight of m/z = 350, which could correspond to a monounsaturated $C_{25}H_{50}$ hydrocarbon or to an aldehyde such as (Z)-17-tetracosenal.

DISCUSSION

As mentioned earlier, this is the first time that the 7-methyl-8-hexadecenoic acid (1) has been isolated. The closest structure reported in the literature has been the isolation of the isomeric 7-methyl-7-hexadecenoic acid from sperm whale oils, which have been a common source of these 7-methyl branched fatty acids (15). In analogy to other similar structures, we probably can speculate at this point that the stereochemistry at the Δ8 double bond could be *cis* but, in any instance, the 7-methyl-8-hexadecenoic acid isolated in this work presents interesting possibilities for stereochemical modifications. For example, the four possible stereoisomers could be synthesized and tested for biological activity, especially pheromone-type interactions. Probably, as is the case of the analogous acid isolated in this work, i.e., the 14-methyl-8-hexadecenoic acid (2), the corresponding alcohol could be expected to provide a more powerful pheromone type of activity (16). As to the biosynthetic origin of this acid, we can only speculate until the necessary biosynthetic experiments are performed. It is very likely that the Δ8 double bond in 7-methyl-8-hexadecenoic acid is introduced after chain elongation and introduction of the 7-methyl group, i.e., the 7-methylhexadecanoic acid is biosynthesized first followed by double bond introduction. Until the latter experiments are performed, we can only speculate about the origin and function of this interesting phospholipid fatty acid.

The corresponding fatty acid methyl ester and alcohol of the 14-methyl-8-hexadecenoic acid (2) have been known for some time to be the active constituents of the sex attractant of both *Trogoderma inclusum* and *Trogoderma granarium* (8), but as to its function in *D. anchorata,* we can only speculate. We should mention here that the

stereochemical features of the natural pheromone are (R)-14-methyl-*cis*-8-hexadecenoate (17). The most likely biosynthetic route for the 14-methylhexadecenoic acid (2) should involve prior biosynthesis of the common 14-methylhexadecanoic acid followed by double bond introduction at Δ8. The origin of terminal 14-methyl branching in short-chain fatty acids has been studied thoroughly (12). The terminal anteiso moiety in fatty acids is generally derived from the branched amino acid isoleucine (12).

The 3-methylheptadecanoic acid is quite rare in nature. In fact, we do not know of any marine source for this acid, and this is the first time that it has been isolated from a marine sponge. However, the latter compound has been shown to be a major component of the overcrowding factors of mosquito larvae, and it possesses larvicidal activity against the southern house mosquito *Culex pipiens quinquefasciatus* (18). The radiolabeled 3-methylheptadecanoic acid also has been useful for heart fatty acid metabolic studies and imaging (18). The [1-^{14}C]β-methylheptadecanoic acid has been used for autoradiographic studies of myocardial infarction while the [1-^{11}C] acid has been useful in positron imaging studies in dog heart (19). Presently, we are synthesizing these fatty acids to test their possible biological activity as a function of stereoisomerism. Work is also in progress trying to elucidate the biosynthetic origin of the isolated acids in this work. We also are studying how the concentration of these acids changes with season.

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REFERENCES

1. Morales, R.W., and Litchfield, C. (1977) *Lipids 12,* 570–576.
2. Litchfield, C., Greenberg, A.J., Noto, G., and Morales, R.W. (1976) *Lipids 11,* 567–570.
3. Carballeira, N., and Maldonado, L. (1986) *Lipids 21,* 470–471.
4. Carballeira, N., Maldonado, L., and Porras, B. (1987) *Lipids 22,* 767–769.
5. Walkup, R.D., Jamieson, G.C., Ratcliff, M.R., and Djerassi, C. (1981) *Lipids 16,* 631–646.
6. Sano, Y. (1967) *Yukagaku 16,* 605–610.
7. Rezanka, T., Vokoun, J., Slavicek, J., and Podojil, M. (1983) *J. Chromatogr. 268,* 71–78.
8. Rodin, J., Silverstein, R., Burkholder, W., and Gorman, J. (1969) *Science 165,* 904–906.
9. Privett, O.S., Dougherty, K.A., Erdahl, W.L., and Stolywho, A. (1973) *J. Am. Oil Chem. Soc. 50,* 516–520.
10. Carreau, J.P., and Dubacq, J.P. (1978) *J. Chromatogr. 151,* 384–390.
11. Andersson, B.A., and Holman, R.T. (1974) *Lipids 9,* 185–190.
12. Carballeira, N., Thompson, J.E., Ayanoglu, E., and Djerassi, C. (1986) *J. Org. Chem. 51,* 2751–2756.
13. Andersson, B.A. (1978) *Prog. Chem. Fats Other Lipids 16,* 279–308.
14. Djerassi, C., Theobald, N., Kokke, W.C., Pak, C.S., and Carlson, R.M.K. (1979) *Pure Appl. Chem. 51,* 1815–1828.
15. Pascal, J.C., and Ackman, R.G. (1975) *Lipids 10,* 478–482.
16. Levinson, H.Z., and Levinson, A.R. (1974) *Naturwissenschaften 61,* 685–686.
17. Mori, K. (1973) *Tetrahedron Lett. 39,* 3869–3872.
18. Hwang, Y-S., Navvab-Gojrati, H.A., and Mulla, M.S. (1978) *J. Agric. Food Chem. 26,* 557–560.
19. Elmaleh, D.R., Livni, E., Levy, S., Strauss, H., and Brownell, G. (1982) in *Nucl. Med. Biol. Adv., Proc. World Congr.* (Claude, Raynaud, ed.) pp. 1682–1683, Pergamon, Oxford, U.K.

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The Composition of Furan Fatty Acids in the Crayfish

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Capillary gas chromatography-mass spectrometry (GC-MS) analysis of the sterol ester fatty acid methyl esters of the crayfish hepatopancreas revealed the presence of at least 30 kinds of unusual furan fatty acids (F acids), which accounted for 28.49% of the total sterol ester fatty acids. On the other hand, only small amounts were found in triacylglycerols (0.5%) and phospholipids (0.7%). Among the F acids, 17 acids were the hitherto unknown ones, the major component being 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoic acid (F_6) (41.83% of the total F acids). These novel acids possessed chain lengths of C_{12} to C_{22}, with the largest concentration at C_{20} (45.38%), C_{18} (41.97%) and C_{16} (10.35%). Odd-numbered F acids also were found, though in a very small amount (0.4%). In the living things other than the crayfish, the longest chain F acid (C_{24}) was detected in the sterol ester of the carp hepatopancreas. The bullfrog, an amphibian, and the turtle, a reptilian, were found to have F acids as well in their livers.

Olefinic furan fatty acids, which were detected by GC-MS, were found to have resulted during the analytical process from cyclodehydration of the diketo-ene formed by autoxidation of the F acids.
***Lipids 23,* 694–700 (1988).**

A furan fatty acid (abbreviated to F acid) was found for the first time in the seed oil by Morris et al. (1), and a series of F acids was detected in some fish by Glass et al. (2–4), Gunstone et al. (5,6) and Scrimgeour (7). The latter acids differed on the point of being tri- or tetra-substituted derivatives from the former di-substituted one, and were recognized mainly as the moieties of cholesteryl ester in the liver and triacylglycerol in the testis. One of the tri-substituted acid (14) (F_2, Table 1) was found in the rubber latex as triacylglycerol (8), and a short-chain tetra-substituted F acid (2) (Table 1) was seen in the soft coral (9). Very recently, two types of dibasic-substituted F acids were reported by Spiteller et al., which were extracted from the blood and urine of men and rats and regarded as oxidative products of the F acids at alkyl substituents of the structure in their living bodies (10–18).

Previously, we reported that the crayfish *Procambarus clarkii* had seven F acids (F_0–F_6, Table 1) in the hepatopancreas (19). This paper describes the results of further elaborated studies on the crustacean lipid. The investigations revealed an additional 23 F acids, among which 17 newly found ones were included (Table 1). The subjects of examinations for the F acids were extended to salmon, carp, ayu sweetfish, loach, yamame, lobsters, locusts, bullfrogs and turtles (Table 3).

MATERIALS AND METHODS

Materials. Crayfish, *P. clarkii,* were caught in October 1984 in Inbanuma in Chiba prefecture near Tokyo. The animals (male), 50–70 g, were maintained in tap water at room temperature for at least two wk, and were fed dried sardines. Then, the animals were killed, and the hepatopancreas was removed. Carp (male, 1300 g), *Cyprinus caprio,* were caught in Inbanuma in July 1984, maintained in tap water for two days, and killed. Removed hepatopancreas was not free of some adipose tissue. Salmon (male), *Oncorhynchus keta,* were captured in the Izumida River in Fukushima prefecture in October 1985; ayu sweetfish (male), *Plecoglossus altivelis,* in the Kano River in Shizuoka prefecture in July 1986; and locust (male and female), *Oxya. chinensis,* in Ibaraki prefecture in October 1985. Yamame, *Oncorhynchus masou,* were donated by the Tokyo metropolitan Suisansikenjyo Okutamabunjyo, Ome, in October 1985, and lobster (male), *Panulicus japonicus,* were obtained at a food market in Tokyo in July 1983. Bullfrog, *Rana catesbeiana,* and turtle, *Clemmys japonica,* were obtained from a local supplier in October 1986. These animals were frozen immediately after being killed, and within 24 hr, tissues were removed and extracted.

Lipid extraction, lipid fractionation and preparation of fatty acid methyl esters. The total lipids of tissues of animals were extracted by the method of Folch et al. (20). Fractionation of the total lipids into sterol esters, triacylglycerols and phospholipids was performed by silica gel column chromatography. The sterol esters were eluted with n-hexane/benzene (2:1, v/v), the triacylglycerols with chloroform/benzene (1:1, v/v), and the phospholipids with methanol/chloroform (2:1, v/v). The total lipids were kept in chloroform solution at −40 C until they were used in the experiment. The methyl esters were prepared by transmethylation with 0.5 M sodium methoxide in methanol at 50 C in screw-cap test tubes for 20 min and purified by thin layer chromatography (TLC) (silica gel 60, 0.25 mm thickness, Merck No. 5715) by developing with n-hexane/benzene (1:1, v/v).

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). GC was carried out with a Hewlett-Packard 5790A with a flame ionization detector (FID), on a Carbowax 20 M fused silica capillary column (25 m × 0.32 mm i.d., Hewlett-Packard), programmed from 150 to 220 C at 4 C/min. The injection and detector were held at 250 and 300 C, respectively. Peak area percentages were obtained with a Hewlett-Packard integrator 3390A. The carrier gas was helium at a flow rate of 1 ml/min. The split ratio was 20:1.

GC-MS was performed with a Hewlett-Packard 5995A, on Carbowax 20 M fused silica capillary column (25 m × 0.32 mm i.d.) with the same temperature programs as used for GC. All mass spectra were obtained at 70 eV ionization electron energy and a source temperature of 180 C.

Mass spectrometry (direct insertion). The mass spectra of the diketo-enes, **36** and **40**, were measured with a JEOL JMS D-300 (inlet temperature 170 C, 70 eV), and of the diketones, **39** and **41**, with a Hewlett-Packard 5995A (inlet temperature 150 C, 70 eV).

High performance liquid chromatography (HPLC) analysis. HPLC was carried out with a Shimadzu LC-6A and a Shimadzu SPD-6A variable wave length UV

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Abbreviations: GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

detector. A Shim-packed CLC-ODS (6 mm × 150 mm, Shimadzu) was used with elution by a 15 min linear gradient of acetonitrile/water (80:20 to 100:0, v/v) at a flow rate of 1.5 ml/min. The effluent was monitored at 234 nm.

Catalytic hydrogenation of fatty acid methyl esters in sterol esters using Lindlar catalyst. Whole fatty acid methyl esters (200 mg) obtained from sterol esters were hydrogenated in n-hexane at an atmospheric pressure over Lindlar catalyst (30 mg) (21). The reaction was followed by measuring the disappearance of the unsaturated fatty acids by GC-MS every 30 min. After three hr, the GC-MS analysis showed that only unsaturated fatty acids were saturated and the furan ring was not hydrogenated under these conditions.

Autoxidation of F_3 methyl ester on the surface of silica gel TLC. A solution of synthetic F_3 methyl ester (50 mg) in n-hexane was applied on the silica gel TLC plate (the same as that used above, Merck No. 5715) that was rinsed with acetone and dried at 100 C for 1.5 hr, developed with n-hexane/benzene (1:1, v/v) up to 3 cm from the upper edge. After the development, the F_3 methyl ester had been spread over about 70 cm^2 of the surface of TLC. Then the plate was left to stand in the air and light (by normal room lamps) at room temperature for one hr. The band, located by spraying with 2′,7′-dichlorofluorescein methanolic solution, was scraped out and extracted with CH_2Cl_2 to give a colorless oil. Fifty-six percent of the starting material (estimated by GC) had disappeared. HPLC analysis of the mixture indicated that the major product was the diketo-ene (**36**), accompanied with a small amount of olefinic F acids (**32**) (**33**). The diketo-ene (**36**) purified by alumina TLC (20 × 20 cm, 0.25 mm thickness, Type E, Merck No. 5713, benzene/ether = 20:1, v/v) produced a colorless oil, 18 mg, which revealed a purity of 80% by HPLC analysis (Fig. 3). On the other hand, purification by silica gel TLC was unsuccessful, owing to its instability on the surface. As described above, this oxidation must be carried out in the light, and in the dark the reaction was slow. When a small amount of F_3 methyl ester (20 mg) was applied on the TLC (spread over about 50 cm^2), 73% of the starting material had reacted. **36**: IR (neat) 1735 ($COOCH_3$), 1685 (C=O), 1605 (C=C) cm^{-1}; UV_{max}(n-hexane) 227 (conjugation) nm; MS (direct insertion, 70 eV) m/z (rel. intensity): 352 (M^+, 48), 334 ($M^+ - H_2O$, 68), 321 ($M^+ - OCH_3$, 28), 305 (21), 303 (12), 295 ($M^+ - (CH_2)_3CH_3$, 55), 291 (47), 281 ($M^+ - (CH_2)_4CH_3$, 4), 277 (13), 263 ($M^+ - (CH_2)_3CH_3 - CH_3OH$, 100), 253 ($M^+ - CO(CH_2)_4CH_3$, 1), 249 ($M^+ - (CH_2)_4CH_3$-$CH_3OH$, 15), 221 ($M^+ - CO(CH_2)_4CH_3$-$CH_3OH$, 3), 205 (28), 195 ($M^+ - (CH_2)_7COOCH_3$, 90), 191 (50), 181 ($M^+ - (CH_2)_8COOCH_3$, 25), 179 (14), 177 (29), 161 (9), 153 ($M^+ - CO(CH_2)_8COOCH_3$, 4), 149 (12), 135 (30), 123 (10), 109 (13).

Synthesis of cyclopentenones (37) (38) from the diketo-ene (36). The diketo-ene (**36**) was converted to cyclopentenones (**37**) (**38**) by the method of Schödel and Spiteller (22), except to use trimethylsilyldiazomethane in place of diazomethane for methylation of carboxylic acid (23) (Scheme 1). Mass spectra of **37** and **38** were identical with those in the literature. **37**: MS m/z (rel. intensity): 352 (M^+, 8), 334 ($M^+ - H_2O$, 4), 321 ($M^+ - OCH_3$, 1), 303 (321 - H_2O, 6), 281 ($M^+ - C_5H_{11}$, 22), 249 (281 - CH_3OH, 22), 196 ($M^+ - CH_2$=$CH(CH_2)_5COOCH_3$, 14), 137 (249 - $(CH_2)_7CO$, 72), 126 (196 - C_5H_{10}, 100). **38**: MS m/z (rel. intensity): 352 (M^+, 13), 334 ($M^+ - H_2O$, 3), 321 ($M^+ - OCH_3$, 5), 303 (321 - H_2O, 4), 296 ($M^+ - C_4H_8$, 2), 278 (296 - H_2O, 4), 181 ($M^+ - (CH_2)_8COOCH_3$, 100), 126 (296 - CH_2=$CH(CH_2)_6COOCH_3$, 65).

Catalytic hydrogenation of the diketo-ene (36). The diketo-ene (**36**) (11 mg) was dissolved in 10 ml ethanol and PtO_2 (3 mg) was added. The mixture was vigorously stirred under hydrogen gas at an atmospheric pressure for 13 hr (Scheme 1). Hydrogenation of **36** was very slow and yielded a complex mixture. Silica gel TLC with n-hexane/ether (3:2, v/v) of the resulting mixture showed the major spots of the dimethyl-1, 4-diketone (**39**) (R_f = 0.4) and F_3 methyl ester (R_f = 0.9). The mass spectrum of **39** isolated by the silica gel TLC (20 × 20 cm, 0.25 mm thickness, n-hexane/ether = 3:2, v/v) was similar to that of the monomethyldiketone, **10**, 13-dioxo-11-methyloctadecanoate (**41**), reported in the literature with an appropriate mass unit shift (24). The compound (**39**) readily converted to F_3 methyl ester with 14% BF_3 in methanol (25). **39**: MS (direct insertion, 70 eV) m/z (rel. intensity): 354 (M^+, 0.5), 336 ($M^+ - H_2O$, 6), 323 ($M^+ - OCH_3$, 9), 251 ($M^+ - (CH_2)_4CH_3$-CH_3OH, 13), 199 ($CO(CH_2)_8COOCH_3$, 13), 198 ($M^+ - CH_2$=CH-$(CH_2)_5COOCH_3$, 6), 183 ($M^+ - (CH_2)_8COOCH_3$, 11), 155 ($M^+ - CO(CH_2)_8COOCH_3$, 29), 139 ($(CH_2)_8COOCH_3 - CH_3OH$, 22), 99 ($CH_3(CH_2)_4CO$, 100).

Conversion of the diketo-ene (36) to olefinic F acid methyl esters (32) (33) during GC analysis. The diketo-ene (**36**), which was confirmed to be free of olefinic F acid methyl esters (**32**) (**33**) by HPLC, was dehydrated to four isomeric olefinic F acid methyl esters during GC analysis under conditions described above (Fig. 4). The retention times and mass spectra of the larger peaks "c" and "d" in Figure 4 were essentially the same as those of **32** and **33**, respectively, in Figure 1B. On the other hand, the mass spectra of the smaller peaks "a" and "b" were basically identical with those of "d" and "c," respectively, suggesting that a pair of two peaks "b" and "c" represent *cis-trans* isomers of the olefinic F acid methyl ester (**32**), and the other pair of "a" and "d," of the olefinic F acid methyl ester (**33**). Peaks "c" and "d" with the longer retention times were *trans* isomers of **32** and **33**, respectively (28).

Oxidation of F_2 methyl ester with m-chloroperbenzoic acid. To a solution of synthetic F_2 methyl ester (114 mg, 0.356 mmol) and $NaHCO_3$ (40 mg) in 15 ml of CH_2Cl_2 at 0 C under N_2 gas was added a solution of m-chloroperbenzoic acid (80 mg, 0.462 mmol, Wako Pure Chemicals, Osaka, Japan, about 70%) in 5 ml of CH_2Cl_2. After being stirred in an ice bath for 10 min, the reaction mixture was washed with 5% $Na_2S_2O_3$, 5% $NaHCO_3$ and brine and dried over anhydrous Na_2SO_4. Removal of solvent gave a yellow oil, which was purified by alumina TLC (20 × 20 cm, 0.25 mm thickness, Merck No. 5713) using benzene-ether (20:1, v/v). Extraction with CH_2Cl_2 gave the diketo-ene derivative of F_2 methyl ester (**40**) (97 mg, 81%), which revealed a purity of 96% by HPLC analysis. **40**: MS (direct insertion, 70 eV) m/z (rel. intensity): 338 (M^+, 9), 320 ($M^+ - H_2O$, 20), 307 ($M^+ - OCH_3$, 22), 291 (3), 289 (10), 281 ($M^+ - (CH_2)_3CH_3$, 3) 277 (10), 249 ($M^+ - (CH_2)_3CH_3$-CH_3OH, 10), 239 ($M^+ - CO(CH_2)_4CH_3$, 29), 235 ($M^+ - (CH_2)_4CH_3$-CH_3OH, 40), 207 ($M^+ - CO(CH_2)_4CH_3$-CH_3OH, 12), 191 (20), 181 ($M^+ - (CH_2)_7COOCH_3$, 42), 177 (7), 167 ($M^+ - (CH_2)_8COOCH_3$, 100), 165 (14), 163 (18), 139 ($M^+ - CO(CH_2)_8COOCH_3$, 23), 123 (31), 121 (30), 111 (60);

IR (neat) 1735 ($COOCH_3$), 1690 (C=O), 1605 (C=C) cm^{-1}; UV_{max}(n-hexane) 226 (conjugation) nm.

The diketo-ene (40) was gradually obtained by autoxidation of F_2 methyl ester on the surface of silica gel TLC as well.

Catalytic hydrogenation of the diketo-ene (40). The diketo-ene (40) (13 mg) in 10 ml ethanol was hydrogenated using PtO_2 (5 mg) under hydrogen gas at an atmospheric pressure for four hr. The resulting mixture was chromatographed on silica gel TLC (20 × 20 cm, 0.25 mm thickness) using n-hexane/ether (3:2, v/v) to give the diketone (41) as a colorless oil, 9 mg. The mass spectrum of 41 was essentially the same as the literature data (24). The compound (41) was cyclodehydrated to F_2 methyl ester with 14% BF_3 in methanol in good yield (88%) (24). 41: MS (direct insertion, 70 eV) m/z (rel. intensity): 340 (M^+, 2), 322 ($M^+ - H_2O$, 4), 309 ($M^+ - OCH_3$, 8), 237 ($M^+ - (CH_2)_4CH_3$-CH_3OH, 12), 199 ($CO(CH_2)_8COOCH_3$, 53), 184 (CH_2=CH-$(CH_2)_5COOCH_3$, 69), 169 ($M^+ - (CH_2)_8COOCH_3$, 46) 141 ($CH_3CHCH_2CO(CH_2)_4CH_3$, 91), 139 ($(CH_2)_8COOCH_3$-CH_3OH, 76), 128 (56), 99 ($CO(CH_2)_4CH_3$, 100).

RESULTS AND DISCUSSION

Capillary GC-MS analysis of the fatty acid methyl esters derived from the sterol ester fraction in the crayfish hepatopancreas revealed several new F acid peaks apart from F_0–F_6 compounds (Fig. 1A) in comparison with the previous packed column data (19). To investigate in detail, the methyl esters were hydrogenated with Lindlar catalyst (21) and followed by urea fractionation (26). The concentrated F acid mixture was analyzed by GC (Fig. 1B) and GC-MS. The spectra showed 30 peaks of F acids (Table 1). Thirteen compounds of them already had been reported, **8, 9, 14, 17, 19, 23, 24** (F_0–F_6) in fish (2–7) and crayfish (19), **7, 15, 25, 29, 30** in fish (3–6 and 12) and a short chain length (2) in the soft coral (9), respectively. But the other 17 acids, **1, 3, 4, 5, 6, 10, 11, 12, 13, 16, 18, 20, 21, 22, 26, 27** and **28**, were newly discovered and identified by GC-MS from the basic data previously reported about F_0–F_6 acids (19). The total content of F acids in the sterol ester was 28.49%. On the other hand, only small amounts were found in triacylglycerols (0.5%) and phospholipids (0.7%).

The other type of F acids **32, 33, 34** and **35** with characteristic mass spectra (Fig. 2) was detected (Fig. 1A, B). These acids were identified by GC-MS as F_3 or F_6 methyl ester with an additional double bond, conjugated with a furan ring (named as olefinic F acids). The former two peaks, **32** and **33**, were derived from F_3, and the latter two, **34** and **35**, from F_6.

Glass et al. (3,27) and Gunstone et al. (6) reported the presence of these olefinic acids in the fish oil but did not

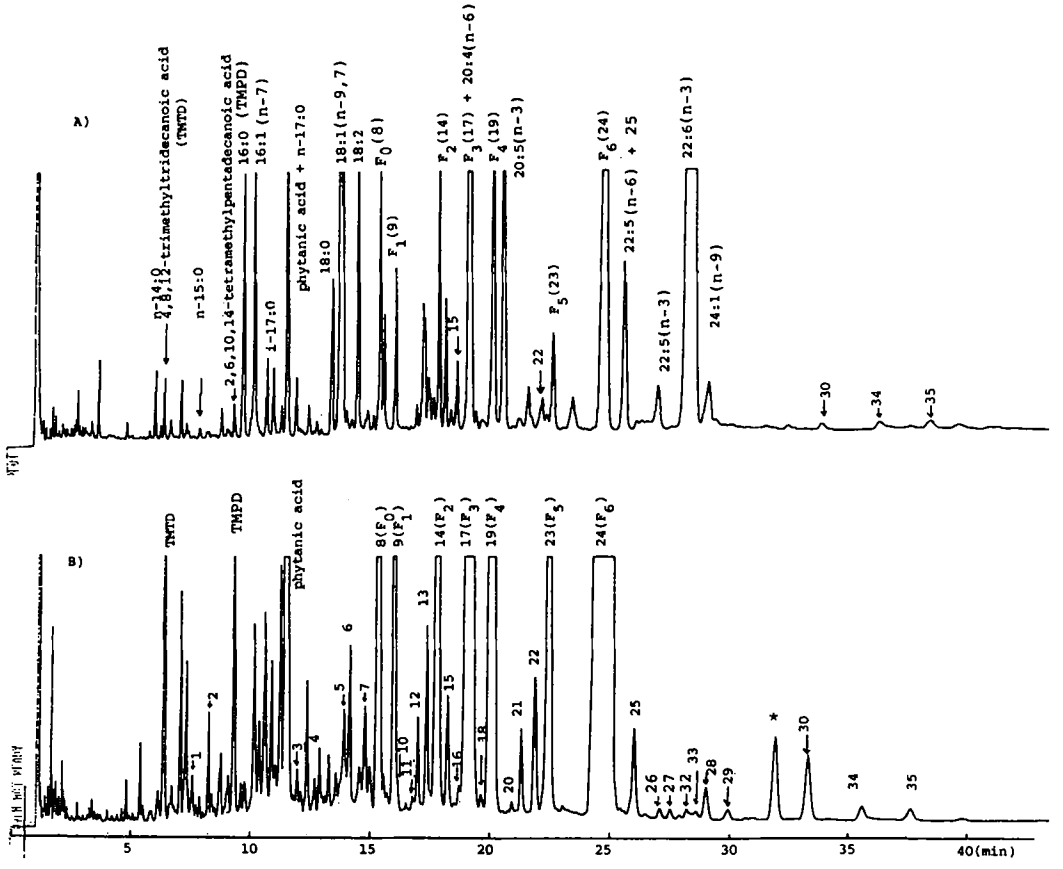


FIG. 1. A) Capillary gas chromatogram of fatty acid methyl esters from crayfish hepatopancreas. B) Fatty acid methyl esters following hydrogenation and urea fractionation. See Table 1 for identities of numbered peaks. *, Phthalate ester.

TABLE 1

Furan Fatty Acid Composition of Sterol Esters[a] from Crayfish Hepatopancreas

Peak no.	ECL[b]	CCL[c]	m	n	R	Wt. % of total fatty acids	Peak no.	ECL	CCL	m	n	R	Wt. % of total fatty acids
1*	15.09	12	2	4	H	0.02	18*	21.38	19	8	5	H	0.03
2	15.42	12	2	4	CH_3	0.04	19 (F_4)	21.51	18	10	2	CH_3	3.45
3*	17.32	14	4	4	CH_3	trace[d]	20*	21.97	19	8	5	CH_3	trace
4*	17.79	14	6	2	CH_3	trace	21*	22.12	19	9	4	CH_3	0.06
5*	18.33	16	4	6	H	0.09	22*	22.36	19	10	3	CH_3	0.11
6*	18.46	16	6	4	H	0.09	23 (F_5)	22.51	20	10	4	H	0.93
7	18.80	16	8	2	H	0.03	24 (F_6)	23.20	20	10	4	CH_3	11.91
8 (F_0)	19.07	16	6	4	CH_3	1.76	25	23.66	20	12	2	CH_3	0.09
9 (F_1)	19.34	16	8	2	CH_3	0.98	26*	23.93	21	10	5	CH_3	0.02
10*	19.56	17	8	3	H	0.02	27*	24.06	21	11	4	CH_3	0.02
11*	19.95	17	6	5	CH_3	0.02	28*	24.40	22	10	6	H	0.04
12*	20.06	17	7	4	CH_3	0.04	29	24.60	22	12	4	H	0.02
13*	20.23	17	8	3	CH_3	0.10	30	25.26	22	12	4	CH_3	0.11
14 (F_2)	20.44	18	8	4	H	1.64	32	24.24		olefinic F_3[e]			0.02
15	20.72	18	10	2	H	0.07	33	24.34					0.02
16*	20.98	18	6	6	CH_3	0.02	34	25.61		olefinic F_6[e]			0.11
17 (F_3)	21.09	18	8	4	CH_3	6.78	35	25.96					0.13

[a]Represents 18.9% of the total lipids of hepatopancreas.
[b]Equivalent chain length.
[c]Carbon chain length.
[d]Less than 0.01%.
[e]F_3 or F_6 methyl ester with one additional double bond, conjugated with a furan ring.
*The acid is unknown.

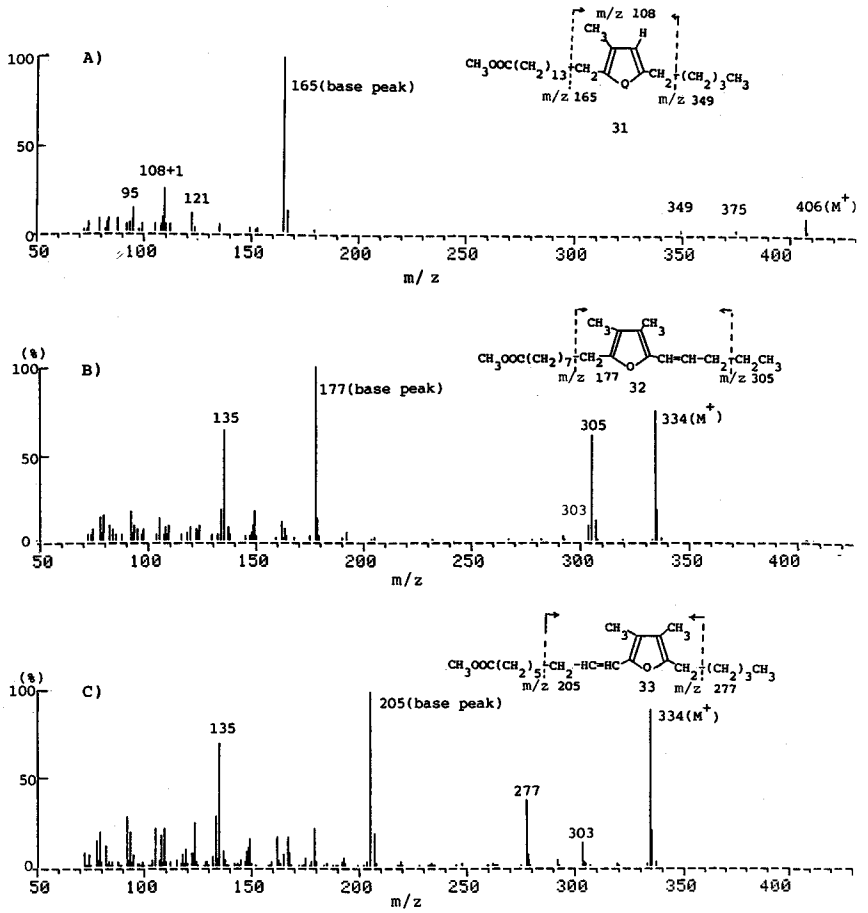
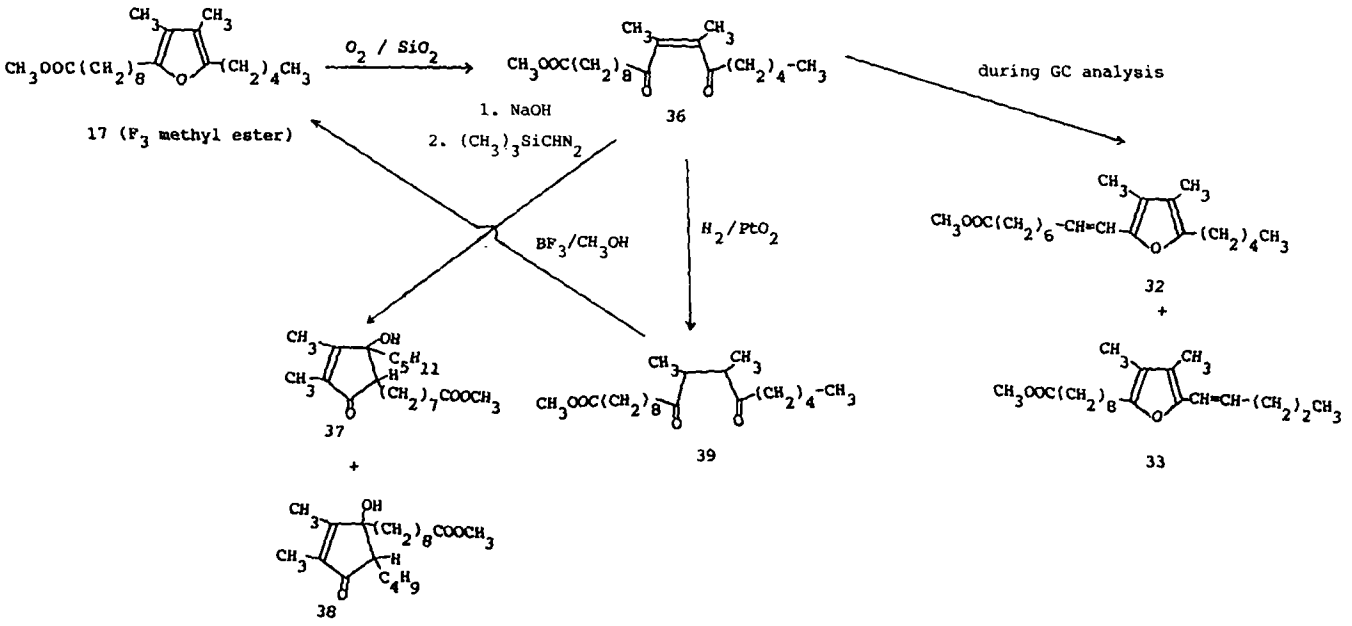


FIG. 2. A) Mass spectra of the long chain F acid methyl ester (31), B) olefinic F acid methyl ester (32), and C) olefinic F acid methyl ester (33).



SCHEME 1. Autoxidation of F_3 acid methyl ester to the diketo-ene (36) on the surface of silica gel TLC. The derivatizations of 36 to cyclopentenones (37) (38) and 1,4-diketone (39), and conversion of 36 to olefinic F acids (32) (33) during GC analysis.

refer to detailed points. We found that synthetic F_3 readily underwent autoxidation on the surface of silica gel TLC to afford the diketo-ene (36), though unaffected on the surface of alumina TLC (Scheme 1). The diketo-ene (36) was not stable enough to be isolated in pure state, as pointed out in the literature (22). But the analysis by HPLC showed that the sample purified by alumina TLC had been separated from olefinic F acids (32) (33) and unchanged F_3 (17). The diketo-ene (36) thus obtained was about 80% in purity by HPLC (Fig. 3). The structure of 36 was established on the basis of spectral data (IR, UV and MS) and chemical derivatizations (Scheme 1). The IR spectrum showed bands at 1685 (C=O) and 1605 (C=C) cm^{-1}, and the UV spectrum had only a maximum at 227 (conjugation) nm. The major mass spectrum fragments of 36 were comparable with their counterparts of the more stable monomethyldiketo-ene (40) derived from F_2 methyl ester (14) (Table 2).

The diketo-ene (36) cyclized in aqueous NaOH solution to give cyclopentenones (37) (38), the structure of which were established by Schödel and Spiteller (22) (Scheme 1). In addition, 36 was hydrogenated using PtO_2 in ethanol to yield the 1,4-diketone (39), which was readily converted to F_3 methyl ester with BF_3 in methanol (25) (Scheme 1). In conclusion, these results described above supported that 36 had a diketo-ene structure.

During GC analysis, the diketo-ene (36) was found to be converted to four isomeric olefinic F acids, with small amounts of various unidentified products (Fig. 4). The retention times and mass spectra of the larger peaks "c" and "d" in Figure 4 were fully consistent with those of the peaks 32 and 33, respectively, in Figure 1. These findings suggested that olefinic F acids, shown in Figure 1, were artifacts that during GC analysis, were produced by cyclodehydration of the diketo-enes formed by

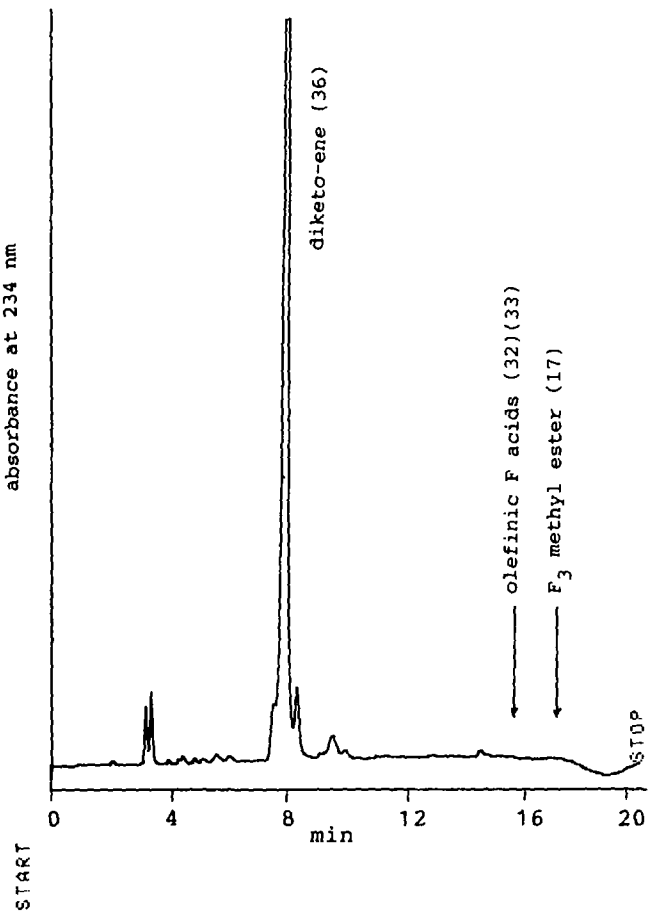


FIG. 3. HPLC of the diketo-ene (36) purified by alumina TLC. The chromatographic conditions are described in the text.

TABLE 2

Comparison of Major Mass Spectral Fragments of Dimethyldiketo-ene (36) with Those of Monomethyldiketo-ene (40)

Fragments	36 (R=CH_3) m/z (rel. int.)[a]	40 (R=H) m/z (rel. int.)[a]
M^+	352 (48)	338 (9)
$M^+ - H_2O$	334 (68)	320 (20)
$M^+ - OCH_3$	321 (28)	307 (22)
a	295 (55)	281 (3)
a − CH_3OH	263 (100)	249 (10)
b	281 (4)	267 (0)
b − CH_3OH	249 (15)	235 (40)
c	252 (1)	239 (29)
c − CH_3OH	221 (3)	207 (12)
d	153 (4)	139 (23)
e	181 (25)	167 (100)
f	195 (90)	181 (42)
g[b]	177 (29)	163 (18)
h[c]	205 (28)	191 (20)

[a]Relative intensity.

[b]The fragments corresponding to base peaks of olefinic F acids with a double bond in the alkyl chain (Fig. 2B).

[c]The fragments corresponding to base peaks of olefinic F acids with a double bond in the alkylcarboxyl chain (Fig. 2C).

TABLE 3

Furan Fatty Acid Compositions[a] of Carp, Salmon, Loach, Bullfrog and Turtle

	Carp (male)			Salmon (male)		Loach (mix)		Bullfrog (male)		Turtle (male)	
	Hepatopancreas	Testes		Testes		Whole body		Liver		Liver	
Peak no.	SE[b] (2.5%)[c]	SE (0.9%)	TG[b] (51.4%)	SE (0.7%)	TG (23.6%)	SE (1.5%)	TG (64.5%)	SE (5.8%)	TG (62.1%)	SE (15.2%)	TG (61.0%)
7	—	—	0.01	—	0.05	—	—	—	—	—	—
8 (F_0)	—	—	—	—	—	—	—	—	—	—	0.19
9 (F_1)	0.02	0.21	0.66	0.14	0.08	—	0.27	1.71	0.69	0.05	0.19
14 (F_2)	0.09	—	0.24	—	0.47	—	0.75	0.14	0.08	—	0.11
15	—	—	0.15	—	0.35	—	—	—	—	—	—
17 (F_3)	—	0.61	0.43	—	0.02	—	0.14	8.00	0.80	0.27	0.58
19 (F_4)	3.20	9.40	3.41	1.30	4.68	—	0.59	—	—	0.09	0.15
21*	0.01	0.01	—	—	—	—	—	—	—	—	—
22*	0.03	0.03	—	—	—	—	—	—	—	—	—
23 (F_5)	0.82	0.32	1.07	0.28	2.98	0.74	0.87	—	—	—	—
24 (F_6)	5.02	15.56	5.39	4.31	9.22	3.02	0.72	1.05	0.51	0.29	0.97
25	—	0.14	0.02	—	0.37	—	—	—	—	—	—
29	0.07	—	0.04	—	0.08	—	—	—	—	—	—
30	0.26	—	0.02	—	0.52	—	—	—	—	—	—
31*[d]	0.03	—	—	—	—	—	—	—	—	—	—
Total	9.55	26.31	11.44	6.03	18.82	3.76	3.34	10.90	2.08	0.70	2.19

[a]Wt. % of total fatty acids of individual lipid class.

[b]SE, sterol esters; TG, triacylglycerols.

[c]Wt. % of total lipids of individual tissue.

[d]Carbon chain length and equivalent chain length of 31 are 24 and 27.85, respectively. For structure of 31, see Fig. 2A.

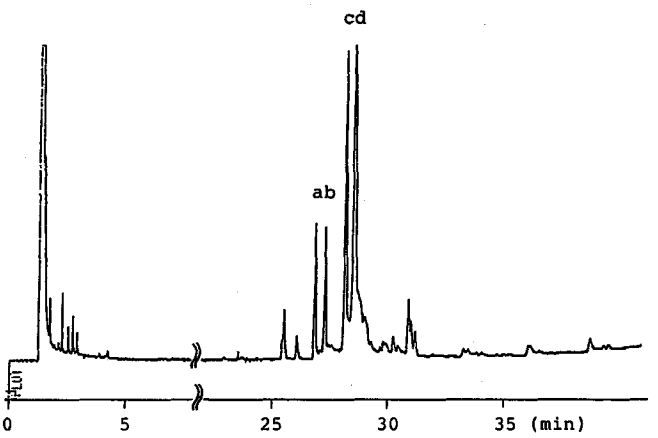


FIG. 4. Conversion of the diketo-ene (36) to olefinic F acids during GC analysis. Peaks "c" and "d" corresponded to 32 and 33, respectively, in Figure 1B. A pair of "a" and "d" was *cis-trans* isomers of 33 and the other pair of "b" and "c," of 32.

autoxidation of F acids under the experimental conditions, mainly on the surface of silica gel TLC.

The carbon chain lengths of the F acids were distributed mainly into 45% of 20 carbon atoms, 42% of 18 carbon atoms followed by 10% of 16 carbon atoms (Table 1). There were also a small amount of odd-numbered acids (10) (11) (12) (13) (18) (20) (21) (22) (26) (27). Their carbon chain lengths ranged from 12 to 22 carbon atoms. The distribution profiles were extremely similar to those of naturally occurring normal fatty acids.

The presence of F acids in the crayfish, one of the crustaceans, prompted us to examine F acids in fish (carp, salmon, ayu sweetfish, loach and yamame), a marine crustacean (lobster), an insect (locust), an amphibian (bullfrog) and a reptilian (turtle) (Table 3). In the carp hepatopancreas and testes, the F acid distribution data were similar to those of fish (4) in order of F_6, F_4 and F_5, but different from those of the crayfish in order of F_6, F_3 and F_5. On the other hand, 31 was a new F acid, having the longest carbon chain of C-24 (Fig. 2). A salmon, which was captured at spawning time, had no detectable F acid (less than 0.01%) in the liver, probably because lipids including F acids had been transported from the liver to the testes at the time. Actually, the testes accumulated F acids as shown in Table 3. Similar facts were reported in brook trout and northern pike by Glass et al. (4). Ayu sweetfish eating mainly river-algae and yamame living in swift streams of inland did not show any special feature of F acids in their organs, and in the lipids of the lobster hepatopancreas (19) and locust (whole body), no F acids were detected. The bullfrog, which belongs to the amphibian family, was found to have F acids in significant amounts in the liver, and its major F acid was F_3, whereas in the fish it always was F_6. Furthermore, the turtle, which belongs to the reptilian family, also contained F acids.

Appreciable quantities of F acids could be observed in animal species besides fish (2-7), including aquatic mollusca (6), and the distribution of the chain lengths presumably parallels that of naturally occurring fatty acids. The functions of such acids in nature are not made clear in spite of active biosynthetic (29), metabolic (12) and physiological studies (18).

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REFERENCES

1. Morris, L.J., Marshall, M.O., and Kelly, W. (1966) *Tetrahedron Lett.*, 4249-4253.
2. Glass, R.L., Krick, T.P., and Eckhardt, A.E. (1974) *Lipids 9*, 1004-1008.
3. Glass, R.L., Krick, T.P., Sand, D.M., Rahn, C.H., and Schlenk, H. (1975) *Lipids 10*, 695-702.
4. Glass, R.L., Krick, T.P., Olson, D.L., and Thorpson, R.L. (1977) *Lipids 12*, 828-836.
5. Gunstone, F.D., Wijesundera, R.C., Love, R.M., and Ross, D. (1976) *J. Chem. Soc. Chem. Commun*, 630-631.
6. Gunstone, F.D., Wijesundera, R.C., and Scrimgeour, C.M. (1978) *J. Sci. Food Agric. 29*, 539-550.
7. Scrimgeour, C.M. (1977) *J. Am. Oil Chem. Soc. 54*, 210-211.
8. Hasma, H., and Subramaniam, A. (1978) *Lipids 13*, 905-907.
9. Groweiss, A., and Kashman, Y. (1978) *Experientia 34*, 299.
10. Spiteller, M., Spiteller, G., and Hoyer, G.A. (1980) *Chem. Ber. 113*, 699-709.
11. Pfordt, J., Thoma, H., and Spiteller, G. (1981) *Liebigs Ann. Chem.*, 2298-2308.
12. Sand, D.M., Schlenk, H., Thoma, H., and Spiteller, G. (1983) *Biochim. Biophys. Acta 751*, 455-461.
13. Thoma, H., Reiner, J., and Spiteller, G. (1984) *J. Chromatogr. 309*, 17-32.
14. Bauer, S., and Spiteller, G. (1985) *Liebigs Ann. Chem.*, 813-821.
15. Rau, O., Spiteller, G., and Bocker, R. (1984) *Liebigs Ann. Chem.*, 1513-1518.
16. Rau, O., Kern, W., and Spiteller, G. (1984) *Liebigs Ann. Chem.*, 1504-1512.
17. Bauer, S., and Spiteller, G. (1985) *Helv. Chim. Acta 68*, 1635-1638.
18. Spitteller, G. (1985) *Iyo Masu Kenkyukai Koenshu 10*, 3-15.
19. Okajima, H., Ishii, K., and Watanabe, H. (1984) *Chem. Pharm. Bul. 32*, 3281-3286.
20. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497-509.
21. Lindlar, H., and Dubuis, R. (1973) *Organic Synthesis*, Coll. Vol. 5 (Baumgarten, H.E., ed.) pp. 880-883, John Wiley and Sons, Inc., New York.
22. Schödel, R., and Spiteller, G. (1985) *Helv. Chim. Acta 68*, 1624-1634.
23. Hashimoto, N., Aoyama, T., and Shioiri, T. (1981) *Chem. Pharm. Bull. 29*, 1475-1478.
24. Lie Ken Jie, M.S.F., and Sinha, S. (1981) *Chem. Phys. Lipids 28*, 99-109.
25. Lie Ken Jie, M.S.F., and Ahmad, F. (1983) *J. Am. Oil Chem. Soc. 60*, 1783-1785.
26. Christie, W.W. (1982) *Lipid Analysis*, 2nd edn., p. 90, Pergamon Press, Oxford.
27. Rahn, C.H., Sand, D.M., Krick, T.P., Glass, R.L., and Schlenk, H. (1981) *Lipids 16*, 360-364.
28. Lie Ken Jie, M.S.F., and Lam, C.M. (1977) *J. Chromatogr. 138*, 446-448.
29. Sand, D.M., Glass, R.L., Olson, D.L., Pike, H.M., and Schlenk, H. (1984) *Biochim. Biophys. Acta 793*, 429-434.

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Rhizomucor miehei Triglyceride Lipase Is Synthesized as a Precursor

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A *Rhizomucor miehei* cDNA library constructed in *Escherichia coli* was screened with synthetic oligonucleotides designed from knowledge of a partial amino acid sequence of the secreted triglyceride lipase (triacylglycerol acylhydrolase EC 3.1.1.3) from this fungus. Lipase-specific recombinants were isolated and their insert sequenced. Unlike characterized bacterial and mammalian triglyceride lipases, the fungal enzyme is synthesized as a precursor, including a 70 amino acid residue propeptide between the 24 amino acid residues of the signal peptide and the 269 residues of the mature enzyme. The precursor processing mechanism, which involves cleavage between a methionine and a serine residue, is unknown. By sequence comparison with other lipases, a serine residue involved in substrate binding was identified in the fungal lipase. The sequence around this residue is well-conserved among characterized lipases. Conservation of an intron in an isolated cDNA recombinant and immunoprecipitation of in vitro synthesized *R. miehei* translation products indicates that the expression of the lipase gene might involve inefficient mRNA splicing.
***Lipids 23,* 701-706 (1988).**

The biosynthesis and activation of extracellular hydrolytic enzymes constitute a problem to secretory cells. Different types of proteases are synthesized as inactive zymogens, presumably to avoid degradation of cell constituents. Among the so far characterized lipases from bacteria and mammals, no zymogen/activated enzyme relationship that could account for a protection mechanism for intracellular or membrane-associated lipids has been described. In the present study, a fungal lipase was chosen as a model for the biosynthesis of lipid-degrading enzymes in lower eukaryotes.

The phycomycete fungus *Rhizomucor miehei* (1) (previously classified as *Mucor miehei* [2]) produces an active extracellular lipase (triacylglycerol acylhydrolase EC 3.1.1.3) that hydrolyzes a broad spectrum of lipids found in animal fat and vegetable oil (3). Recently, we reported the partial purification and characterization of this enzyme (4). The *R. miehei* lipase (RML) was found to be present in fermentation supernatants in a predominant form called RML-A. The RML-A form was converted to the RML-B form during purification involving steps performed at low pH. The two enzyme forms showed a high degree of antigenic identity but differed in a number of characteristics, e.g., in affinity towards concanavalin A. RML-A, with high concanavalin A affinity, required activation at pH 10.5, while the RML-B form, which showed low concanavalin A affinity, did not need this step for maximal lipolytic activity. Therefore, we suggested that RML-B might be activated from RML-A through a partial deglycosylation that could influence the activity toward emulsions (4).

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Abbreviations: RML, *Rhizomucor miehei* lipase; SDS, sodium dodecylsulphate; bp, base pair; HIC, hydrophobic interaction chromatography; HPLC, high performance liquid chromatography; kb, kilobase; LU, lipase units.

Multiple forms of extracellular lipases have been described from fungi (5-7), and this phenomenon apparently is due to both postsecretorial modifications like partial proteolysis and deglycosylation and to synthesis of truly different lipases.

We now report on the final purification and characterization of both enzyme forms, and on the molecular cloning of a corresponding cDNA. We present evidence that this triglyceride lipase is synthesized as a precursor with a signal peptide and a substantial propeptide. Maturation of the RML mRNA precursor apparently involved inefficient splicing.

MATERIALS AND METHODS

Purification of RML-A and RML-B. The two initial steps of RML-A purification by anion exchange chromatography on DEAE Sepharose and affinity chromatography on concanavalin A-Sepharose were carried out as described (4). Further purification was performed by hydrophobic interaction chromatography (HIC) on a TSK-Phenyl-5PW column. The ultrafiltration concentrate from the concanavalin A-Sepharose chromatography was made 1.2 M in Na_2SO_4, and applied at a flowrate of 1 ml/min to the TSK-Phenyl-5PW column (7.5 × 75 mm) mounted with a precolumn of TSK Guard gel Phenyl-5PW (6 × 10 mm). The elution was carried out by a linear gradient from 100% buffer A (1.2 M sodium sulfate in buffer B) to 100% buffer B (20 mM sodium phosphate, at pH 7.0) during 15 min. The separation procedure on the TSK-column was repeated several times by the injection of 350 μl aliquots.

The partially purified RML-B (4) was subjected to gel filtration chromatography on TSK G3000 SW. The RML-B preparation was applied at a flow rate of 1 ml/min as a 1% solution in 0.1 M sodium phosphate (pH 7.0) to the TSK G3000 SW column (7.5 × 600 mm) mounted with a precolumn TSK SWP (7.5 × 75 mm). The 1% solution was injected in volumes of 100 μl. Lipase activity (LU) was measured using tributyrine as substrate according to the procedure of Brockman (8) as modified (4). Determinations of protein (9) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4) were performed as described.

Carbohydrate and amino acid analysis on RML-A and RML-B. Carbohydrate analysis was performed by use of the phenol sulfuric acid method (10) with glucose as a standard. Amino acid analysis was performed on a Beckman Amino Acid Analyzer 121 MB. Half-cystine was determined as the S-β-(4-pyridylethyl)-derivative after reduction with tributylphosphine and coupling with 4-vinylpyridine (11,12). One sample was treated with 4-vinylpyridine without prior reduction for the determination of potential free cysteines.

Partial amino acid sequence determination of RML-A and RML-B. RML-A was digested with *Armillaria mellea* protease (13), with an enzyme to substrate ratio of 1:40 (mol:mol), and the resulting fragments were separated by high performance liquid chromatography (HPLC). To identify suitable fragments for the construction of

oligonucleotide probes, only peptides that showed a high ratio between 280 nm and 214 nm were sequenced because these fragments could contain tryptophan and/or tyrosine. Sequence determination was carried out (14) both on the native enzymes (amino-terminal sequence) and on selected suitable fragments by the use of a Gas Phase Sequencer (Applied Biosystems Model 470 A).

Nucleic acid procedures. A cDNA library that had been used to isolate a cDNA encoding an aspartic proteinase from *R. miehei* (15) was used to isolate RML-specific recombinants. The library was constructed on total cellular mRNA. Oligonucleotides were used in colony screening and primer extension experiments as described (16–18). The methods used for DNA sequencing (19), cell-free translations followed by immunoprecipitations (16) and mRNA blot analysis (15) have been described. Oligonucleotides were synthesized on an Applied Biosystems model 380 A DNA synthesizer.

RESULTS

Purification of RML-A and RML-B and partial amino acid sequence. The purification of RML-A and RML-B are summarized in Table 1. The elution pattern from the HIC of RML-A on the TSK-Phenyl-5PW column showed two main peaks (data not shown). The first peak appeared to contain practically all the lipase activity. Fractions corresponding to this lipase peak were collected from 18 identical runs, and the enzyme was desalted and concentrated (ca. 13 times) by ultrafiltration. The purification of RML-B by gel filtration on the TSK G3000 SW column is shown in Figure 1. The peak corresponding to a volume of elution of 23.43 ml contained the lipase activity. Fractions corresponding to this peak were collected from six identical runs, and the pooled protein material was concentrated (ca. 10 times) by ultrafiltration. The purified RML-A and RML-B were analyzed by SDS-PAGE. Both lipases migrated as a single band with the same apparent molecular weights of ca. 32 kD (Fig. 2). The purified RML-A also was analyzed by gel filtration on a TSK G3000

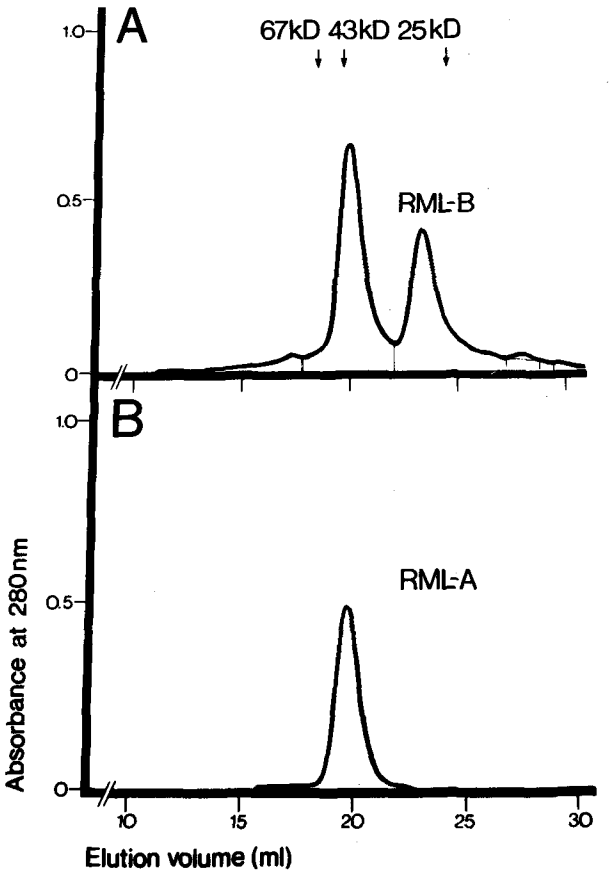

FIG. 1. Gel filtration chromatography of RML-A and RML-B on a TSK G3000 SW column. Panel A: Partially purified RML-B from the Phenyl-Sepharose chromatography. Panel B: Purified RML-A. The arrows indicate the elution positions of the m.w. standards: serum albumin (67 kD), ovalbumin (43 kD) and chymotrypsinogen A (25 kD). The elution positions of the RML-A and RML-B correspond to molecular weights of 42.5 kD and 27.5 kD, respectively. The protein eluting at a position corresponding to a molecular weight of 42.5 kD in panel A is an aspartic proteinase secreted by *R. miehei* (15).

TABLE 1

Purification of RML-A and RML-B

Purification steps for RML-A	% Yield (step-wise) based on LU-values	Protein content[a]	Spec. activity LU/mg prep.	Spec. activity LU/mg protein
Crude powder	100	42%	157	374
DEAE-sepharose chromatography	74.5	90%	1170	1300
ConA-sepharose chromatography	49.2	6.8 mg/ml		3618
HIC on TSK-Phenyl-5PW	98.5	5.6 mg/ml		8260

Purification steps for RML-B	Total protein[a] mg	Total activity LU	Yield %	Specific activity LU/mg
Phenyl Sepharose chromatography[c]	6.0	18000	100	3000
GFC on TSK G3000 SW	1.8[b]	13500	75	7500

[a]According to Lowry et al. (9).

[b]The UF-concentrate contained 2 mg protein/ml.

[c]Ref. 4.

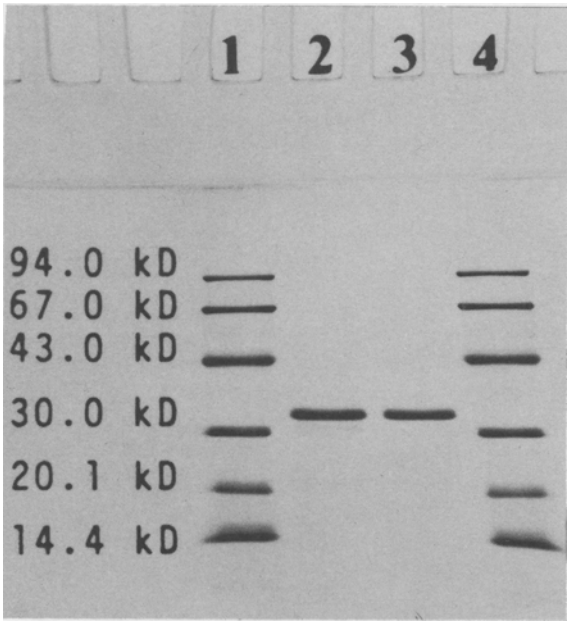

FIG. 2. SDS-PAGE of purified RML-A and RML-B. Lanes 1 and 4: m.w. standards; Lane 2: RML-A (2.7 μg). Lane 3: RML-B (3.4 μg). The gel was stained with Coomassie Brilliant Blue. The apparent molecular weights of both RML-A and RML-B are 32 kD.

SW column (Fig. 1) and eluted as a single peak with an apparent molecular weight of ca. 42.5 kD. RML-B had an apparent molecular weight of ca. 27.5 kD as determined by gel filtration (Fig. 1). The carbohydrate contents of the purified lipases were 11% (w/w) for RML-A and 4% (w/w) for RML-B.

The following N-terminal amino acid sequence was determined on both the A and B form of RML: Ser-Ile-Asp-Gly-Gly-Ile-Arg-Ala-Ala-Thr-Ser-Gln-Glu-Ile-Asn-Glu-Leu-Thr-Tyr-Tyr-Thr-. The following sequence was identified in one of the tryptophan-containing fragments isolated from the proteolytic digest of RML-A: Arg-Thr-Val-Ile-Pro-Gly-Ala-Thr-Trp-Asp. This sequence was used for the design of an RML-specific oligonucleotide mixture.

Molecular cloning and sequence of RML cDNA. An *R. miehei* cDNA library (15) was screened with an RML-specific oligonucleotide mixture, which had the following sequence: 5′ ($\frac{A}{G}$TCCCCANGTNGCNCC)3′. This oligonucleotide mixture is specific for the RML-mRNA in a region encoding the pentapeptide Gly-Ala-Thr-Trp-Asp, corresponding to the described proteolytic fragment of the purified RML-A. Hybridization and initial washing of the filters were done at 43 C. After autoradiography, the filters were washed at 47 C. Colonies that still showed strong hybridization were isolated, and the inserted cDNAs were sequenced. Two such recombinants, p353.7 and p353.16, had inserts of 1.2 kb. The DNA sequence obtained from these two recombinants both started in the middle of a signal peptide (Fig. 3), and extended through to the poly(A) tail. A third recombinant, p566, was found to contain 74 additional nucleotides inserted in the coding region (Fig. 3). Because the isolated recombinants did not include sequence for the 5′ part of a signal peptide with its initiating methionine codon, a synthetic oligonucleotide 5′(CGAGAGGGGATGAGGGGTGG)3′ was synthesized. This oligonucleotide is complementary to the RML

```
        -1 1
ATCAGAATC ATG GTT CTC AAG CAG CGT GCA AAC TAT CTG GGC TTT CTG ATT GTA TTC TTC   51
          MET VAL LEU LYS GLN ARG ALA ASN TYR LEU GLY PHE LEU ILE VAL PHE PHE  -78

ACG GCG TTC CTG GTC GAA GCC GTG CCA ATC AAG AGA CAA TCA AAC AGC ACG GTG GAT AGT  111
THR ALA PHE LEU VAL GLU ALA VAL PRO ILE LYS ARG GLN SER ASN SER THR VAL ASP SER  -58

CTG CCA CCC CTC ATC CCC TCT CGA ACC TCG GCA CCT TCA TCA TCA TCA CCA AGC ACA ACC GAC  171
LEU PRO PRO LEU ILE PRO SER ARG THR SER ALA PRO SER SER SER PRO SER THR THR ASP  -38

CCT GAA GCT CCA GCC ATG AGT CGC AAT GGA CCG CTG CCC TCG GAT GTA GAG ACT AAA TAT  231
PRO GLU ALA PRO ALA MET SER ARG ASN GLY PRO LEU PRO SER ASP VAL GLU THR LYS TYR  -18

GGC ATG GCT TTG AAT GCT ACT TCC TAT CCG GAT TCT GTG GTC CAA GCA ATG AGC ATT GAT  291
GLY MET ALA LEU ASN ALA THR SER TYR PRO ASP SER VAL VAL GLN ALA MET SER ILE ASP    3

GGT GGT ATC CGC GCT GCG ACC TCG CAA GAA ATC AAT GAA TTG ACT TAT TAC ACT ACA CTA  351
GLY GLY ILE ARG ALA ALA THR SER GLN GLU ILE ASN GLU LEU THR TYR TYR THR THR LEU   23

TCT GCC AAC TCG TAC TGC CGC ACT GTC ATT CCT GGA GCT ACC TGG GAC TGT ATC CAC TGT  411
SER ALA ASN SER TYR CYS ARG THR VAL ILE PRO GLY ALA THR TRP ASP CYS ILE HIS CYS   43

GAT GCA ACG GAG GAT CTC AAG ATT ATC AAG ACT TGG AGC ACG CTC ATC TAT GAT ACA AAT  471
ASP ALA THR GLU ASP LEU LYS ILE ILE LYS THR TRP SER THR LEU ILE TYR ASP THR ASN   63

GCA ATG GTT GCA CGT GGT GAC AGC GAA AAA ACT ATC TAT ATC GTT TTC CGA GGT TCG AGC  531
ALA MET VAL ALA ARG GLY ASP SER GLU LYS THR ILE TYR ILE VAL PHE ARG GLY SER SER   83

TCT ATC CGC AAC TGG ATT GCT GTA AGT ATC CAT ACA GAG CGC TCT TTC ACT TTG TGT ATG  591
SER ILE ARG ASN TRP ILE ALA VAL SER ILE HIS THR GLU ARG SER PHE THR LEU CYS MET (103)

TAGTTCGTCTCATTTGATGCAACCTTTCAATGTAG GAT CTC ACC TTT GTG CCA GTT TCA TAT CCT CCG  659
TERM                               ASP LEU THR PHE VAL PRO VAL SER TYR PRO PRO  101

GTC AGT GGT ACA AAA GTA CAC AAG GGA TTC CTG GAC AGT TAC GGG GAA GTT CAA AAC GAG  719
VAL SER GLY THR LYS VAL HIS LYS GLY PHE LEU ASP SER TYR GLY GLU VAL GLN ASN GLU  121

CTT GTT GCT ACT GTT CTT GAT CAA TTC AAG CAA TAT CCA AGC TAC AAG GTT GCT GTT ACA  779
LEU VAL ALA THR VAL LEU ASP GLN PHE LYS GLN TYR PRO SER TYR LYS VAL ALA VAL THR  141

GGT CAC TCA CTC GGT GGT GCT ACT GCG TTG CTT TGC GCC CTG GGT CTC TAT CAA CGA GAA  839
GLY HIS SER LEU GLY GLY ALA THR ALA LEU LEU CYS ALA LEU GLY LEU TYR GLN ARG GLU  161

GAA GGA CTC TCA TCC AGC AAC TTG TTC CTT TAC ACT CAA GGT CAA CCA CGG GTA GGC GAC  899
GLU GLY LEU SER SER SER ASN LEU PHE LEU TYR THR GLN GLY GLN PRO ARG VAL GLY ASP  181

CCT GCC TTT GCC AAC TAC GTT GTT AGC ACC GGC ATT CCT TAC AGG CGC ACG GTC AAT GAA  959
PRO ALA PHE ALA ASN TYR VAL VAL SER THR GLY ILE PRO TYR ARG ARG THR VAL ASN GLU  201

CGA GAT ATC GTT CCT CAT CTT CCA CCT GCT GCT TTT GGT TTT CTC CAC GCT GGC GAG GAG 1019
ARG ASP ILE VAL PRO HIS LEU PRO PRO ALA ALA PHE GLY PHE LEU HIS ALA GLY GLU GLU  221

TAT TGG ATT ACT GAC AAT AGC CCA GAG ACT GTT CAG GTC TGC ACA AGC GAT CTG GAA ACC 1079
TYR TRP ILE THR ASP ASN SER PRO GLU THR VAL GLN VAL CYS THR SER ASP LEU GLU THR  241

TCT GAT TGC TCT AAC AGC ATT GTT CCC TTC ACA AGT GTT CTT GAC CAT CTC TCG TAC TTT 1139
SER ASP CYS SER ASN SER ILE VAL PRO PHE THR SER VAL LEU ASP HIS LEU SER TYR PHE  261

GGT ATC AAC ACA GGC CTC TGT ACT TAA GAAATACCAGTTATACGATATGTAGGAAGTAGTATTTTTTAGG 1209
GLY ILE ASN THR GLY LEU CYS THR TERM

AAGAGATTTATATGTATTAAACAAATATATATATATATACCGCTGCGCGAGAACCTGTATT POLYA
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FIG. 3. Sequence of the preproRML cDNA together with the deduced amino acid sequence given by three-letter abbreviations. The horizontal lines show the positions of oligonucleotides used for cDNA-library screening and primer extension. An arrow shows the processing position used in maturation of RML. Nucleotides are numbered from the first base in the initiating Met codon, and amino acids are numbered from the first residue in the mature RML. Two short horizontal arrows indicate the 5′ and 3′ splice sites of the 74 nucleotides long intervening sequence. Amino acid Met-103 is the carboxyterminal residue in a short lipase precursor variant. A pentapeptide around the Ser-residue involved in substrate binding is boxed.

mRNA in a region encoding the amino acid sequence Pro-Pro-Leu-Ile-Pro-Ser-Arg underlined in the propeptide region in Figure 3. After this oligonucleotide had been labeled to a high specific activity with T4 polynucleotide kinase and ^{32}P-γ-ATP, it was used in a primer extension reaction on *R. miehei* mRNA. The primer extension reaction products were electrophoresed on a 10% polyacrylamide/urea gel and two RML-specific cDNA products, one 150 nucleotides long and the other one 160 nucleotides long, were both electroeluted and sequenced.

Structure of the RML precursor. DNA sequence data obtained from cDNA clones p353.7, p353.16 and p566, and from sequencing of the 5′ end of the RML mRNA through specific primer extension as described above, is compiled in Figure 3. Apart from the presence in the cDNA recombinant p566 of 74 extra basepairs, the sequences obtained from the different isolated plasmids and from the primer extension products were in total agreement. From the cDNA sequence data, it can be concluded that RML is synthesized as a part of a 363 amino acids

long precursor with a molecular weight of 39,529. The first 24 amino acid residues constitute a typical hydrophobic signal peptide. According to the predictive rules of von Heijne (20), the signal peptide is cleaved from the subsequent precursor by a cleavage between the alanine and valine residues at positions −71 and −70. Because the amino terminal sequence analysis of purified RML identified Ser-Ile-Asp-Gly-Gly- as the amino-terminus of the active enzyme, the lipase precursor has a 70 amino acid residues-long propeptide. Beginning with the amino-terminal serine residue (number 1 in Fig. 3), the mature lipase extends through 269 residues. The molecular weight of the protein backbone of the mature enzyme is 29,472.

In addition to the described preproRML, a potential variant lipase precursor was identified. A cDNA recombinant, p566, was found to contain 74 additional nucleotides inserted at a position between the codons for Ala-90 and Asp-91 (Fig. 3). This 74-nucleotide insert has the hexanucleotide GTAAGT at its 5′ end and the trinucleotide TAG at its 3′ end. Taking this structure, which is typical for intervening sequences, and the fact that the fragment was present in one cloned cDNA recombinant and absent from two others, this fragment most probably represents the result of differential splicing. A lipase precursor translated from such an intron-containing transcript would be only 197 amino acid residues long due to the presence of an inframe termination codon within the intervening sequence. After posttranslational processing of the signal and propeptides, only 103 residues would be left in this short, mature lipase-related variant. The molecular weight of this precursor and its maturation product would be 21,592 and 11,535, respectively.

After immunoprecipitation by the use of lipase specific antiserum of the primary translation products from *R. miehei* mRNA-supplemented rabbit reticulocyte lysate (Fig. 4), lipase precursors with molecular weights of 40 kD and 22 kD were identified. These two precursors, which corresponded well to the RML precursors suggested by cDNA sequencing, were precipitated in roughly equimolar amounts and therefore indicate that the 74 bp intron is conserved in roughly half of the translatable lipase mRNA.

Structure of RML mRNA. Poly(A)$^+$ mRNA was analyzed after blotting onto nitrocellulose with a ^{32}P-labeled RML-specific cDNA probe. An RML-specific transcript of 1.3 kb (Fig. 5) was identified by this analysis. The size of this mRNA is in good agreement with the length of the sequenced cDNA (Fig. 3). Due to the limitations of gel resolution of polyadenylated mRNA, the hybridizing mRNA may contain both the intron containing (74 nucleotides extra) as well as the spliced variant of RML mRNA. The RML-specific cDNA obtained by primer extension with the propeptide region specific oligonucleotide 5′ (CGAGAGGGGATGAGGGGTGG)3′ were 150 nucleotides and 160 nucleotides long (data not shown). If these cDNA represent full-length reverse transcripts, the cap site of the RML mRNA will be only 20 to 30 nucleotides 5′ to the initiating methionine codon. In the 3′ end of the mRNA, a 104-nucleotides long 3′ untranslated region was found. This region contains no typical polyadenylation signal (AATAAA) as found on most eukaryotic mRNA. At a position 22 nucleotides upstream from the poly(A) tail, a repetitive structure consisting of seven times the dinucleotide TA is localized.

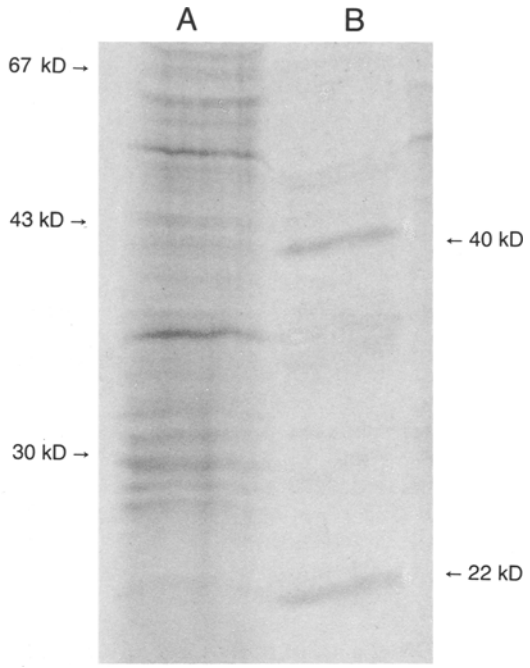

FIG. 4. SDS-polyacrylamide gel electrophoresis, followed by autoradiography of *in vitro* translation products and their immunoprecipitates. Total mRNA extracted from *R. miehei* was translated in a rabbit reticulocyte lysate with L-[^{35}S]methionine (lane A) and immunoprecipitated with rabbit antisera against RML (lane B). Two primary translation products were precipitated. The position of molecular weight standards are shown to the left.

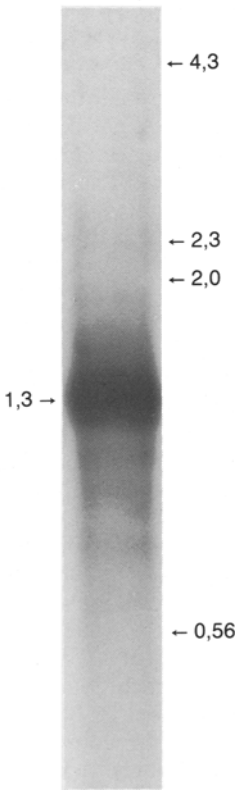

FIG. 5. RNA blot analysis of *R. miehei* poly(A)$^+$ RNA. Five μg of mRNA were separated on a 1.2% agarose gel, blotted to nitrocellulose and hybridized with nick-translated RML cDNA. The nucleotide length of *Hin*dIII digested phage lambda DNA fragments is indicated.

DISCUSSION

The triglyceride lipase from *R. miehei* can be purified as two enzyme forms: RML-A and RML-B. RML-A was isolated when neutral pH was maintained during all purification steps. In contrast, RML-B seems to be generated only when the preparation was exposed to low pH (~4.5) during purification. Both forms show the same apparent molecular weight (32 kD) on SDS gels after reduction. By gel filtration, the A- and B-forms eluted at positions corresponding to molecular weights of 42.5 kD and 27.5 kD, respectively.

The discrepancy between the molecular weights by gel filtration and SDS-PAGE of the A- and B-forms might be due to different apparent molecular size or shape of the enzyme forms in native and denatured conditions. The fact that RML-A and RML-B have identical apparent molecular weights determined by SDS-PAGE, whereas RML-B has a lower apparent molecular weight than RML-A by gel filtration, also could be explained by different molecular size/shape of their native forms. Determination of the total amount of carbohydrate in the purified A- and B-forms indicated a significant difference. Thus, the B-form seems to be less glycosylated than the A-form, which might give rise to the retardation observed by gel filtration (Fig. 1). Consequently, it seems likely that RML-B is generated as a result of low pH-dependent (~4.5) deglycosylation of RML-A. Amino acid composition (Table 2) of the A- and B-forms show that the two enzymes have a similar protein backbone, which, within experimental error, is confirmed by the data obtained through cDNA sequencing.

The possibility of the A- and B-forms being the results of different amino-terminal processing of a common precursor is excluded by the fact that both forms had the same amino-terminal as determined by amino acid sequencing. The fact that three independent cDNA clones all had exactly the same DNA sequence (except for the intron in p566) suggests that both lipase forms are encoded by one gene and therefore have the same primary structure.

Primary structures of triglyceride lipases from bacteria and mammals have been reported. These lipases show a large variation in the length of the protein chain. The shortest lipase has been reported from *Pseudomonas fragi* (21) containing only 135 amino acid residues. Two other bacterial lipases from *Staphylococcus aureus* (22) and *Staphylococcus hyicus* (23) have 690 (including signal peptide) and 603 residues, respectively. Mammalian triglyceride lipases have protein chains with intermediary sizes of 472 (rat hepatic lipase [sequence given in Ref. 27]), 449 (rat lingual lipase [24]), and 377 (porcine pancreatic lipase [25]) residues. The related enzyme, human lecithin-cholesterol acyltransferase (26) has 416 residues. The only regions with significant homology between these lipases and the 269 residues of mature RML are shown in Figure 6, which also includes a region from the human lipoprotein lipase (27). This alignment identifies one serine residue, located in a stretch of hydrophobic amino acid residues, as totally conserved. In porcine pancreatic lipase, this serine residue has been identified as an essential part of a hydrophobic attachment site involved in lipase adsorption to the water-insoluble triglyceride-substrate interfaces (28).

It should be noted that the primary structure of RML deduced from cDNA sequencing has 7 cysteine residues. Analysis by the use of 4-vinylpyridine for determination of potential free cysteines (Table 2) revealed traces of free cysteines in both enzyme forms. In proteins in which all cysteine residues are part of disulfide bridges, this analysis gives a clearly negative result. Therefore, it is likely that RML has a cysteine residue that is not involved in disulfide bridge formation and that is, therefore, free or involved in other as yet unknown bindings.

In none of the so far reported triglyceride lipases (most of them deduced from DNA sequence) has a zymogen been identified. The precursor of RML as deduced from

TABLE 2

Amino Acid Composition of RML-A and RML-B

Amino acid	RML-A	RML-B	Deduced from cDNA
Asp/Asn	27.7	28.7	26
Thr[a]	26.1	26.0	27
Ser[a]	29.1	26.0	25
Glu/Gln	20.6	22.5	21
Pro	19.2	14.3	13
Gly	17.3	20.6	20
Ala	18.9	18.9	18
Cys-SH[b]	5.5 (.37)	5.7 (.21)	7
Val[a]	20.2	21.4	21
Met	2.5	1.3	1
Ile[a]	13.6	15.9	17
Leu	21.5	22.1	22
Tyr	13.8	15.0	15
Phe	8.4	10.1	10
Lys	7.0	7.8	7
His	4.9	5.9	6
Trp	3.2	3.9	3
Arg	9.9	10.1	10
NH_3	24.3	33.1	19
Totals			269

[a]Determined as extrapolated values to 0 or infinite hydrolysis time.

[b]Determined as S-β-(4-pyridylethyl)-cysteine, in parentheses the found amount of potentially free cysteines.

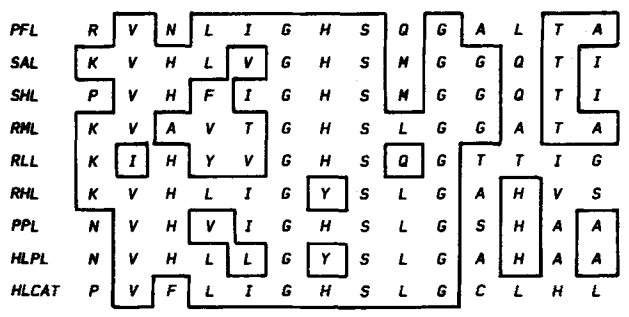

FIG. 6. Homology among substrate binding regions in lipases. Regions from 9 lipases are aligned, and residues are boxed where at least 3 out of 9 possible identities were found. PFL, *Pseudomonas fragi* lipase (21); SAL, *Staphylococcus aureus* lipase (22); SHL, *Staphylococcus hyicus* lipase (23); RML, *R. miehei* lipase; RLL, rat lingual lipase (24); RHL, rat hepatic lipase (sequence from Ref. 27); PPL, porcine pancreatic lipase (25); HLPL, human lipoprotein lipase (27); HLCAT, human lecithin-cholesterol acyltransferase (26). Amino acids are given by their one-letter abbreviations.

the DNA sequence in Figure 3 has a 70 amino acid residues-long propeptide between the typical signal peptide and the amino terminus of the mature enzyme. The maturation involves a proteolytic cleavage between a methionine and a serine residue (Fig. 3). We do not consider it likely that this Met-residue just 5′ to the Ser-residue could be the initiating methionine. If this was the case, the resulting secreted lipase would have no signal peptide, and the length of the identified primary translation products (Fig. 4) could not be accounted for. As the first 24 amino acid residues of the deduced precursor constitutes a typical signal peptide, including two basic residues in its amino terminal end and a long strongly hydrophobic core, we can only conclude that RML is synthesized as a part of a zymogen-like precursor. This observation raises interesting questions as to the temporal events in lipase secretion from a fungal cell. Processing of the 70 amino acid residues-long precursor peptide might involve a step-wise mechanism, because the first five residues end in the typical dibasic target sequence, Lys-Arg (residues −67 and −66 in Fig. 3). Propeptides have been identified in mammalian colipases (see Ref. 29 for an alignment of procolipases from pig and horse), and it is interesting to note that the propeptide in these enzymes are five residues long and read the sequence, Val-Pro-Asp-Pro-Arg. Processing of this colipase propeptide might be an example of proline-directed arginyl cleavage, a processing mechanism that recently has been reviewed (30). The colipase pentapropeptide has three identical residues in homologous positions as compared with the first five residues in the propeptide of RML. Apart from this short region, which may be functionally equivalent in proenzyme processing in proRML and in procolipases, these proteins have no homology.

Out of three isolated RML-specific cDNA recombinants, one (p566) was found to contain a 74 bp intervening sequence. Differential splicing of introns is well-known in higher eukaryotes, and it also has been demonstrated in a few genes from the lower eukaryotes, e.g., the glucoamylase gene of *Aspergillus niger* (16,31). However, no obvious advantages from this extra variation in the mechanism of gene expression can be identified in these two examples from the lower eukaryotes. In the case of RML, we might have observed a phenomenon best characterized as inefficient splicing of a precursor mRNA. The 74 base pair intervening sequence identified in recombinant p566 does not have a sequence showing homology to the internal splicing signal consensus, which is directly involved in lariat formation. A poor fit to this consensus, which in the filamentous fungi reads $\begin{matrix}TG\\AA\end{matrix}CT\begin{matrix}A\\G\end{matrix}AC$, has been suggested as a cause of inefficient mRNA precursor splicing (32) in lower eukaryotes.

It should be stressed that we cannot totally rule out the possibility that the cDNA recombinant p566 might represent a nuclear nonprocessed mRNA species. However, because a substantial fraction of the immunoprecipitable precursors is found as the short variant, we favor a theory of inefficient mRNA splicing, due to a weak internal splicing signal in the intron. We do not believe that the short lipase variant encoded by this intron-containing mRNA could have any lipase activity, simply because the substrate binding site would not be included in the molecule. During purification of the enzyme, we have not at any step observed such a short variant with lipase activity. If it is synthesized by the fungus in vivo, it might be unstable due to as yet unidentified mechanisms that eliminate aberrant protein molecules.

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REFERENCES

1. Schipper, M.A.A. (1978) *Studies in Mycology,* No. 17, pp. 53-71, Institute of the Royal Netherlands Academy of Sciences and Letters, Amsterdam, The Netherlands.
2. Cooney, D.G., and Emerson, R. (1964) *Thermophilic fungi,* pp. 17-27, W.H. Freeman and Company, San Francisco, CA.
3. Moskowitz, G.J., Cassaigne, R., West, I.R., Shen, T., and Feldman, L.I. (1977) *J. Agric. Food Chem. 25,* 1146-1150.
4. Huge-Jensen, B., Galluzzo, D.R., and Jensen, R.G. (1987) *Lipids 22,* 559-565.
5. Iwai, M., and Tsujiska, Y. (1974) *Agric. Biol. Chem. 38,* 1241-1247.
6. Iwai, M., Tsujiska, Y., and Tominaga, Y. (1979) *Agric. Biol. Chem. 43,* 893-900.
7. Benzonana, G. (1974) *Lipids 9,* 166-172.
8. Brockman, H.L. (1981) *Methods Enzymol. 71,* 619-627.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
10. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. (1956) *Anal. Chem. 28,* 350-356.
11. Rüegg, U. Th., and Rudinger, J. (1974) *Israel J. Chem. 12,* 391-401.
12. Friedman, M., Krull, L.H., and Cavins, J.E. (1970) *J. Biol. Chem. 245,* 3868-3871.
13. Moody, A.J., Thim, L., and Valverde, I. (1984) *FEBS Lett. 172,* 142-148.
14. Thim, L., Hansen, M.T., and Sørensen, A.R. (1987) *FEBS Lett. 212,* 307-312.
15. Boel, E., Beck, A-M., Randrup, K., Dræger, B., Fiil, N.P., and Foltmann, B. (1986) *Proteins 1,* 363-369.
16. Boel, E., Hjort, I., Svensson, B., Norris, F., Norris, K.E., and Fiil, N.P. (1984) *Eur. Mol. Biol. Org. J. 3,* 1097-1102.
17. Boel, E., Vuust, J., Norris, F., Norris, K., Wind, A., Rehfeld, J.F., and Marcker, K.A. (1983) *Proc. Natl. Acad. Sci. USA 80,* 2866-2869.
18. Gergen, J.P., Stern, R.H., and Wensink, C. (1979) *Nucl. Acids Res. 7,* 2115-2135.
19. Maxam, A., and Gilbert, W. (1980) *Methods Enzymol. 65,* 499-560.
20. von Heijne, G. (1986) *Nucl. Acids Res. 14,* 4683-4690.
21. Kugimiya, W., Otani, Y., Hashimoto, Y., and Takagi, Y. (1986) *Biochem. Biophys. Res. Commun. 141,* 185-190.
22. Lee, C.Y., and Iandolo, J.J. (1984) *J. Bacteriol. 166,* 385-391.
23. Götz, F., Popp, F., Korn, E., and Schleifer, K.H. (1985) *Nucl. Acids Res. 13,* 5895-5906.
24. Docherty, A.J.P., Bodmer, M.W., Angal, S., Verger, R., Riviero, C., Lowe, P.A., Lyons, A., Emtage, J.S., and Harris, T.J.R. (1985) *Nucl. Acids Res. 13,* 1891-1903.
25. De Caro, J., Boudouard, M., Bonicel, J., Guidoni, A., Desnuelle, P., and Rovery, M. (1981) *Biochim. Biophys. Acta 671,* 129-138.
26. McLean, J., Fielding, C., Drayna, D., Dieplinger, H., Baer, B., Kohr, W., Henzel, W., and Lawn, R. (1986) *Proc. Natl. Acad. Sci. USA 83,* 2335-2339.
27. Wion, K.L., Kirchgessner, T.G., Lusis, A.J., Schotz, M.C., and Lawn, R.M. (1987) *Science 235,* 1638-1641.
28. Guidoni, A., Benkouka, F., De Caro, J., and Rovery, M. (1981) *Biochim. Biophys. Acta 660,* 148-150.
29. Sternby, B., Engström, Aa., Hellman, U., Vikert, A.M., Sternby, N-H., and Borgström, B. (1984) *Biochim. Biophys. Acta 784,* 75-80.
30. Schwarz, T. (1986) *FEBS Lett. 200,* 1-10.
31. Boel, E., Hansen, M.T., Hjort, I., Høgh, I., and Fiil, N.P. (1984) *Eur. Mol. Biol. Org. J. 3,* 1581-1585.
32. Ballance, D.J. (1986) *Yeast 2,* 229-236.

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Effects of Hyperoxia and Diet on Murine Tissue Levels of Vitamin E and Polyunsaturated Fatty Acids

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One hundred eighty-six adult female mice were studied to examine the effect of manipulating dietary vitamin E and fractional inspired oxygen concentrations (FiO_2) on tissue levels of vitamin E, total polyunsaturated fatty acids (TPUFA) and conjugated dienes (CD) as an index of lipid peroxidation. Animals were fed custom diets containing either 0, 50 or 150 ppm DL-α-tocopheryl acetate. Once plasma vitamin E levels of mice fell below 0.2 mg/dl (at week 19), all mice were placed in chambers containing either room air ($FiO_2 \sim 0.21$) or $FiO_2 > 0.95$ for the next 72 hr. Dietary manipulation had a major impact on the levels of vitamin E in plasma, lung and perirenal adipose tissues ($p < 0.0001$, $p < 0.0001$ and $p < 0.005$, respectively). Dietary vitamin E deprivation was associated with significant reductions in lung glutathione peroxidase (GPX) activities ($p < 0.05$) and in plasma TPUFA levels ($p < 0.05$). No significant effect attributable to either diet or FiO_2 was observed for liver vitamin E, liver TPUFA or lung TPUFA levels, or for those of CD in any tissue examined. Adipose TPUFA levels were depressed in all dietary groups exposed to $FiO_2 > 0.95$, when compared with those of groups exposed to room air. The high FiO_2 exposures also were associated with marked reductions in lung to body weight ratios ($p < 0.01$). These data suggest that dietary vitamin E treatment after long-term feeding can modify vitamin levels in plasma, lung and adipose tissues, and lung GPX activities. Vitamin E levels in liver seemed less responsive to our dietary manipulations in adult female mice, though expressing liver vitamin E levels in terms of TPUFA revealed significant differences between the ratios from 0 and 150 ppm vitamin E groups ($p < 0.05$).
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Whenever animals are exposed to high fractional inspired oxygen concentrations (FiO_2), acute lung injury ensues. The pathophysiological processes include the recruitment and activation of specific cells to the lung and the generation of toxic oxygen products that are thought to be responsible for the damage of lung vasculature and epithelium. In response to this stress, the lung possesses a number of protective mechanisms against the free-radical peroxidizing compounds. These include vitamin E, ascorbic acid, uric acid, ceruloplasmin and a battery of enzymes, some of which rely on glutathione.

The administration of vitamin E to prevent hyperoxia-associated lung injury has been studied in experimental animals (1-9) with conflicting results. Similarly, reports regarding its benefit for infants with respiratory distress consequent to prolonged oxygen therapy (10,11) are conflicting. Some problems inherent in the interpretation of these findings might be resolved by an investigation of the ability of plasma measures to reflect the distribution or content of vitamin E in those tissues during hyperoxic challenge. Kornbrust and Mavis (12) reported that the degree of peroxidation in microsomes prepared from various rat organs was related to vitamin E content, and that the lung and heart were least susceptible to peroxidation because of the high vitamin E to total polyunsaturated fatty acid (TPUFA) ratios; they did not examine, however, plasma ratios or the impact of hyperoxia. Sevanian and coworkers (13) and Poukka Evarts and Bieri (14) also have reported similar differences in vitamin E to TPUFA ratios among these tissues of young rats.

In the present study, we examined the impact of $FiO_2 \sim 0.21$ (room air) and $FiO_2 > 0.95$ (hyperoxia) along with three dietary levels of vitamin E on tissue vitamin E and TPUFA concentrations of adult female mice. Yam et al. (15) found that adult rats succumbed to hyperoxia, because unlike the newborn animals, they did not exhibit induction of certain lung antioxidant enzymes, glutathione peroxidase (GPX), glutathione reductase, superoxide dismutase and others, in response to hyperoxic challenge. Therefore, we also wished to determine whether adult mice fed insufficient dietary vitamin E and exposed to hyperoxia would manifest alterations in lung GPX activities.

METHODS

Animals. One hundred and eighty-six adult female Swiss Webster mice (Harlan/Sprague-Dawley, Indianapolis, IN) weighing ca. 25 g each were placed in stainless steel cages, three or four per cage. The room was temperature and humidity controlled, having equal periods of light and dark (12 hr). For one wk following delivery, mice were fed ad libitum a custom-prepared diet containing 50 ppm DL-α-tocopheryl acetate (ICN Nutritional Biochemicals, Cleveland, OH) and water. Subsequent to this acclimatization period, animals were randomly assigned by cage to receive purified diets (from the same supplier) containing either 0, 50 or 150 ppm DL-α-tocopheryl acetate. Details of the diet composition and growth data have been reported (16). By week 19, mice that were fed 0 ppm vitamin E exhibited marked weight loss and depressed plasma vitamin E levels (<0.2 mg/dl), which is indicative of vitamin E deficiency. At this time, half the mice in each dietary treatment group were exposed to either $FiO_2 > 0.95$ or room air for the next 72 hr under ambient temperatures (22-26 C). The 72-hr period was selected because research by Poland and colleagues suggested that any period longer than this would be accompanied by considerable mortality (17). Chamber atmospheres were continuously filtered and circulated. Oxygen and CO_2 concentrations were monitored several times each day using a Beckman Medical Gas Analyzer LB-2 (Beckman Instruments, Inc., Fullerton, CA). Chambers were opened daily for several min to provide animals with food and water.

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Abbreviations: GPX, glutathione peroxidase; ANOVA, analysis of variance; CD, conjugated dienes; FiO_2, fractional inspired oxygen concentrations; TPUFA, total polyunsaturated fatty acids.

Tissue collections. Following the 72 hr exposures, mice were removed from chambers and anesthesized briefly with methoxyflurane (Pitman-Moore, Inc., Washington Crossing, NJ). No animals died at the end of the 72 hr exposure period, although several mice from the vitamin E deficient group appeared to suffer respiratory distress once they were removed from chambers for killing. Data from seven mice were not included in these analyses because of problems with tissue processing; therefore, 63 mice comprised the 0 ppm group (30 at $FiO_2 > 0.95$), 56 mice were in the 50 ppm group (26 at $FiO_2 > 0.95$) and 63 mice were in the 150 ppm group (33 at $FiO_2 > 0.95$). Blood was obtained following procedures previously described (16). Analyses performed on plasma aliquots represent pooled samples from three to four mice in each cage on the same treatments, specifically, 17 samples for the 0 ppm vitamin E group, 16 samples for the 50 ppm and 18 for the 150 ppm vitamin E group. Whole lungs, livers, perirenal adipose tissues were excised, perfused with saline to remove blood, wrapped in an aluminum foil and frozen at −70 C. Once thawed, all tissues were minced, weighed and homogenized in ice-cold 1% (w/v) EDTA. Adipose tissues from three to four animals in each cage also were pooled before analyses.

Vitamin analyses. Extractions of tissues other than plasma were performed with hexane following saponification as described (18,19). Vitamin E, specifically α- and γ-tocopherols were measured using a Waters model 510 high performance liquid chromatograph (Waters Assoc., Milford, MA) equipped with a Perkin Elmer LC-75 variable UV-visible spectrophotometer (Perkin Elmer, Norwalk, CT). The reversed-phase analytical column was a 3 μm Supelcosil ODS column, 15 cm long × 4.6 mm, i.d. (Supelco, Inc., Bellfonte, PA). The isocratic solvent system consisted of 5% water in absolute methanol (v/v) at a flow rate of 1.2 ml/min. The column effluent was monitored at 292 nm. Calibration curves were established for all compounds of interest from measured peak heights of standards supplied by Bhagavan of Hoffmann La Roche relative to those of the selected internal standard, dl-tocol. Data for α- and γ-tocopherols are given as the sum total of α- and γ-tocopherols (vitamin E), because γ-tocopherol was not detected frequently in any of the tissue samples.

Total polyunsaturated fatty acid (TPUFA) analyses. Aliquots of plasma, lung, liver or adipose homogenates were extracted according to the method of Folch and coworkers (20), redissolved in toluene, and transesterified according to a published procedure (21). Pentadecanoic acid (15:0) was added at the beginning of the extraction to serve as an internal standard. Fatty acids from 14:0 to 22:6ω3 were separated and quantified by gas-liquid chromatography using a Perkin Elmer Sigma 3B chromatograph with a 10 ft long by 2 mm i.d. glass column packed with 3% SP2310 and 2% SP2300 on Chromosorb WAW (Supelco, Inc., Bellefonte, PA). A calibration mixture of fatty acid methyl esters (NuChek, Elysian, MN) and additional standard solutions were injected daily to confirm retention times and to calculate response factors. The instrument was operated with an injection temperature of 210 C, the flame detector at 250 C and the column from 185 to 220 C. Peak heights were electronically calculated by a Hewlett Packard 3390A recording integrator (Avondale, PA). Total PUFA (TPUFA) was defined as the sum of 18:2ω6, 18:3ω3, 20:2ω6, 20:3ω6, 20:4ω6, 20:5ω3, 22:3ω3, 22:4ω6 and 22:6ω3. These definitions were selected to reproduce those originally reported by Poukka Evarts and Bieri (14).

Other measurements. Lung homogenates (in 1% EDTA, w/v) were centrifuged at 105,000 g for one hr so the soluble fraction could be obtained for determination of GPX activity. The activities were measured using H_2O_2 according to the method of Lawrence and Burk (22). Linear regression analyses of log-transformed data were used to calculate activities that were corrected for spontaneous reaction rates. Protein concentration was determined by the method of Lowry et al. (23). DNA content was assessed by the microfluorometric method of Kissane and Robbins (24). GPX activities then were expressed per mg protein and per μg DNA. Analysis of conjugated dienes (CD) as an index of lipid peroxidation was performed in tissue homogenates at 233 nm by the method of Recknagel and Ghoshal (25) as modified by Till and coworkers (26). The amount of dienes was estimated using a molar extinction coefficient of 2.52×10^4 M^{-1} and then expressed in μmoles per mg protein or μmoles per μg DNA. The lung wet weight to body weight ratio was calculated to reflect the extent of lung edema (15) and is referred to as the lung edema index.

Statistical analyses. All data are expressed as mean ±1 SEM. Data were analyzed by two-way analysis of variance (ANOVA) using dietary vitamin E and FiO_2 exposures as factors. Whenever appropriate, one-way ANOVA and Duncan's multiple-range tests available in the statistical package, Statistics for Physical and Social Sciences, $SPSS^X$ (27,28) also were employed. Pearson r correlation and multiple linear regression analyses also were performed using $SPSS^X$.

RESULTS

Dietary treatments had a marked influence upon several components quantified in lung tissue homogenates. After 19 weeks of feeding the 0 ppm vitamin E diet, mice exhibited significantly lower ($p < 0.0001$) lung vitamin E levels than their supplemented counterparts (Table 1). No significant differences were apparent between lungs of mice fed 50 and 150 ppm vitamin E, or between mice on $FiO_2 > 0.95$ and those exposed to room air. Neither diet

TABLE 1

Lung Tissue Vitamin E Concentrations of Adult Female Mice on Three Levels of Dietary Vitamin E and Exposed to Hyperoxic or Normoxic Conditions

Dietary vitamin E levels	Lung homogenate vitamin E[a]		
	$FiO_2 > 0.95$	$FiO_2 \sim 0.21$	Both FiO_2 groups combined[b]
(ppm)	(μg/g wet weight)		
0	7.94 ± 3.8	11.78 ± 5.5	9.95 ± 3.4[c]
50	133.32 ± 16.9	100.59 ± 14.2	115.78 ± 11.0[d]
150	131.69 ± 18.5	177.83 ± 29.5	153.66 ± 17.2[d]

[a]All values are expressed as mean ± SEM.

[b]Significant differences were observed for dietary treatments only. Values in the same column not sharing the same superscript (c, d) are significantly different from each other: $p < 0.0001$.

nor FiO_2 treatments had any significant effect on lung TPUFA levels (Table 2). Dietary vitamin E deprivation resulted in lower lung GPX activities as compared with those from mice on 150 ppm vitamin E (Table 3). No differences in GPX activities were apparent between animals on hyperoxic and normoxic exposures within each dietary group, but considerable variation was observed in those of the 150 ppm dietary group. When GPX activities were expressed per μg DNA, there were no significant differences observed among dietary or FiO_2 groups (data not shown). Lung cell number was not altered by dietary treatments, but hyperoxia produced a significant ($p < 0.05$) reduction in lung cell number in both 0 and 50 ppm groups (data not shown). In contrast, neither treatment affected lung protein levels.

Data for lung CD levels, lung edema index, liver vitamin E and liver TPUFA levels are shown in Table 4. No significant differences in lung CD levels were obtained for either dietary or FiO_2 treatments. Lung edema indices were significantly greater in all dietary treatment groups exposed to hyperoxia as compared with those on room air ($p < 0.01$). Vitamin E and TPUFA levels in liver homogenates precluded detecting any significant effects attributable to dietary or FiO_2 treatments (data not shown).

As observed in lung homogenates, vitamin E levels of adipose tissue homogenates from animals fed the 0 ppm vitamin E were significantly lower ($p < 0.005$) than those of the 50 and 150 ppm dietary groups (Table 5). However, unlike the lung measures, a consistent dose-response trend can be observed for the 0, 50 and 150 ppm dietary groups in both hyperoxic and normoxic treatments. This trend is apparent despite the large variation in vitamin E levels of mice exposed to $FiO_2 > 0.95$ and receiving either 50 or 150 ppm vitamin E. Mean adipose TPUFA levels (g per g wet tissue) were consistenly lower in groups exposed to hyperoxia as compared with those on room air, respectively: for 0 ppm (20.99 ± 9.2 vs 70.26 ± 29.8), 50 ppm (14.78 ± 6.2 vs 37.42 ± 19.6), and 150 ppm dietary vitamin E (10.84 ± 5.6 vs 38.39 ± 12.8). Considerable variability was apparent in TPUFA levels of normoxic groups, though significant differences between FiO_2 groups were observed ($p < 0.05$). In contrast, no statistically significant differences were observed for CD

TABLE 2

Lung Tissue TPUFA Levels of Adult Female Mice on Three Levels of Dietary Vitamin E and Exposed to Hyperoxic or Normoxic Conditions

Dietary vitamin E levels	Lung homogenate TPUFA[a,b]	
	$FiO_2 > 0.95$	$FiO_2 \sim 0.21$
(ppm)	(mg/g wet weight)	
0	15.72 ± 1.7	19.11 ± 2.6
50	18.96 ± 4.0	17.64 ± 1.4
150	15.90 ± 1.8	15.00 ± 1.3

[a]All values are expressed as mean ± SEM.

[b]No significant differences were observed for diet or oxygen treatments nor the interaction.

TABLE 3

Lung Glutathione Peroxidase (GPX) Activity Levels of Adult Female Mice on Three Levels of Dietary Vitamin E and Exposed to Hyperoxic or Normoxic Conditions

Dietary vitamin E levels	Lung GPX[a]		
	$FiO_2 > 0.95$	$FiO_2 \sim 0.21$	Both FiO_2 groups combined[b]
(ppm)	(μmoles NADPH/min/mg protein)		
0	17.26 ± 3.4	9.95 ± 1.8	13.61 ± 3.1[c]
50	20.13 ± 3.0	22.58 ± 5.1	22.50 ± 2.0[c,d]
150	52.69 ± 19.7	28.49 ± 10.6	41.66 ± 11.8[d]

[a]All values are expressed as mean ± SEM.

[b]Significant differences were observed among dietary treatments only. Values not sharing the same superscript (c, d) are significantly different from each other: $p < 0.05$.

TABLE 4

Influence of Dietary Vitamin E and/or FiO_2 Exposure on Lung Tissue Conjugated Diene (CD) Levels, Lung Edema Indices, Liver Tissue Vitamin E and TPUFA Levels of Adult Female Mice[a]

Dietary vitamin E levels	FiO_2 group	Lung CD μmol/mg protein[b,c]	Lung edema index[d]	Liver vitamin E (μg/g)[c]	Liver TPUFA (mg/g)[c]
0	>0.95	488.62 ± 120.5	12.10 ± 0.5	131.56 ± 41.4	176.06 ± 21.0
0	~0.21	288.60 ± 37.7	11.35 ± 0.4	244.89 ± 77.7	187.40 ± 30.3
50	>0.95	420.11 ± 88.7	12.29 ± 0.6	158.29 ± 61.0	194.38 ± 33.8
50	~0.21	430.76 ± 190.5	11.00 ± 0.5	300.46 ± 74.2	176.70 ± 27.5
150	>0.95	405.69 ± 82.4	12.53 ± 0.4	410.40 ± 122.9	206.23 ± 24.8
150	~0.21	511.70 ± 159.7	10.56 ± 0.4	251.34 ± 46.0	179.12 ± 23.7

[a]Values represent mean ± SEM.

[b]There were no differences in values observed among groups with respect to lung protein content.

[c]There were no differences in values observed between FiO_2 groups. Nor was there any significant interaction between diet and FiO_2 treatments.

[d]There were no differences between dietary groups, only between FiO_2 treatments: for all mice on $FiO_2 > 0.95$: 12.31 ± 0.3 vs all mice on $FiO_2 \sim 0.21$: 10.99 ± 0.2, $p < 0.01$.

TABLE 5

Perirenal Adipose Tissue Vitamin E Concentrations of Adult Female Mice on Three Levels of Dietary Vitamin E and Exposed to Hyperoxic or Normoxic Conditions

Dietary vitamin E levels	Adipose homogenate vitamin E[a]		
	$FiO_2 > 0.95$	$FiO_2 \sim 0.21$	Both FiO_2 groups combined[b]
(ppm)	(mg/g wet weight)		
0	11.02 ± 7.0	34.96 ± 28.9	22.99 ± 14.7[c]
50	233.68 ± 156.8	158.53 ± 30.1	193.60 ± 72.5[d]
150	453.80 ± 175.0	352.16 ± 61.6	408.63 ± 99.2[d]

[a]All values are expressed as mean ± SEM.

[b]Only a significant diet effect was found. Values in the same column not sharing the same superscript (c, d) are significantly different from each other: $p < 0.005$.

levels of adipose tissue among dietary or FiO_2 groups (data not shown).

Plasma vitamin E levels were highly correlated with those of lung tissue, $r = +0.68$, $p < 0.0001$. Significant differences in plasma levels were observed among all three dietary treatment groups ($p < 0.0001$), as shown in Table 6. No differences were apparent between FiO_2 groups. In Table 7, plasma TPUFA levels of both hyperoxic and normoxic 0 ppm vitamin E groups were markedly lower than those on 150 ppm vitamin E ($p < 0.05$). Again, no significant differences in plasma levels of CD among any of the groups were observed (data not shown).

Table 8 shows the vitamin E to TPUFA ratios for all tissues examined in these experiments. As observed with tissue vitamin E levels for plasma and adipose, we observed significant differences largely among dietary treatment groups with one exception: lung vitamin E to TPUFA ratios exhibited a significant interaction effect

TABLE 6

Plasma Vitamin E Concentrations of Adult Female Mice on Three Levels of Dietary Vitamin E and Exposed to Hyperoxic or Normoxic Conditions

Dietary vitamin E levels	Plasma vitamin E[a]		
	$FiO_2 > 0.95$	$FiO_2 \sim 0.21$	Both FiO_2 groups combined[b]
(ppm)	(mg/dl plasma)		
0	0.058 ± 0.03	0.034 ± 0.02	0.046 ± 0.02[c]
50	0.512 ± 0.10	0.600 ± 0.11	0.556 ± 0.07[d]
150	0.912 ± 0.18	1.045 ± 0.25	0.971 ± 0.15[e]

[a]All values are expressed as mean ± SEM.

[b]Significant differences were observed between dietary groups only. Values not sharing the same superscript (c, d, e) are significantly different from each other: $p < 0.0001$.

TABLE 7

Plasma TPUFA Concentrations of Adult Female Mice on Three Levels of Dietary Vitamin E and Exposed to Hyperoxic or Normoxic Conditions

Dietary vitamin E levels	Plasma TPUFA[a]		
	$FiO_2 > 0.95$	$FiO_2 \sim 0.21$	Both FiO_2 groups combined[b]
(ppm)	(g/dl plasma)		
0	50.60 ± 12.1	76.86 ± 7.2	64.51 ± 7.4[c]
50	82.15 ± 10.4	105.82 ± 16.9	93.99 ± 10.1[c,d]
150	104.27 ± 13.6	99.07 ± 23.8	101.96 ± 12.6[d]

[a]All values are expressed as mean ± SEM.

[b]Significant differences were observed between dietary groups only: values not sharing the same superscript (c, d) are significantly different from each other: $p < 0.05$.

TABLE 8

Influence of Dietary Vitamin E and/or FiO_2 Exposure on Vitamin E to TPUFA Ratios of Plasma, Lung, Liver and Adipose Tissues in Adult Female Mice

Dietary vitamin levels	FiO_2 group	Vitamin E to TPUFA ratios[a]			
		Plasma[b]	Lung[c]	Liver[d]	Adipose[e]
0	>0.95	1.45 ± 0.9	0.72 ± 0.4	0.73 ± 0.2	0.47 ± 0.4
0	~0.21	0.40 ± 0.3	0.72 ± 0.3	1.21 ± 0.5	0.8 ± 0.5
50	>0.95	7.65 ± 2.2	11.92 ± 2.2	0.79 ± 0.2	6.84 ± 4.1
50	~0.21	6.47 ± 1.1	6.56 ± 1.0	1.36 ± 0.4	16.51 ± 11.4
150	>0.95	9.66 ± 1.6	11.87 ± 2.8	1.85 ± 0.5	35.23 ± 12.8
150	~0.21	15.27 ± 4.5	15.88 ± 2.6	1.44 ± 0.2	14.63 ± 3.8

[a]Values represent mean ± SEM.

[b]Significant differences were found among all three dietary groups for plasma ratios ($p < 0.0001$).

[c]Lung ratios exhibited a significant interaction effect ($p < 0.05$).

[d]A significant dietary effect was observed for liver ratios, with ratios for the 0 ppm group being significantly lower than those of the 150 ppm group ($p < 0.05$).

[e]Significant differences between ratios for the 50 and 150 ppm supplemented groups with those of 0 ppm group were observed ($p < 0.01$). Adipose ratios have been multiplied by 1000.

between dietary and FiO_2 exposures ($p < 0.05$). Hyperoxic mice fed 50 ppm vitamin E had significantly higher ratios than those found for normoxic mice consuming the same level of dietary vitamin E ($p < 0.05$). In addition, in contrast to the lack of observed significant differences with liver vitamin E and TPUFA levels separately among diet groups, the use of the vitamin E to TPUFA ratio for liver homogenates provided at least an indication of dietary manipulation: the ratios for mice on 0 ppm vitamin E were significantly lower than those for mice on the 150 ppm vitamin E diet ($p < 0.05$).

When examining these ratios across tissues from supplemented mice only, plasma and lung ratios were similar in magnitude. Liver ratios were nearly 10-fold lower and adipose, nearly 300 to 1000 times lower than those of plasma or lung. These relative differences for tissue ratios were not observed in tissues of mice fed 0 ppm vitamin E.

DISCUSSION

Plasma vitamin E levels (not plasma vitamin E to TPUFA ratios) were fairly good predictors of lung vitamin E levels in these experiments. FiO_2 treatments may explain some of the remaining variation, but the results of ANOVA analyses did not support this speculation.

Statistical analyses suggest that plasma, lung and adipose tissues respond to the dietary vitamin E regimens, though not to the same magnitude. Differences in affinity, uptake, depletion and degradation of the vitamin among tissues as well as the concentrations of other antioxidant-like compounds and prooxidants may affect the static tissue levels of vitamin E. For example, by expressing hepatic vitamin E levels in terms of those for TPUFA, we observed differences in tissue ratios among dietary treatments. These differences were not observed when tissue vitamin E levels were contrasted across dietary groups.

Dietary vitamin E also appeared to correlate with lung GPX activities (Table 3). This observation is inconsistent with that reported by Chow and coworkers (29); they found no difference in lung GPX activities of adult rats fed 0 or 45 mg vitamin E per kg diet for eight wk. Our results also suggest that GPX is not induced by short-term hyperoxia as observed in newborn rabbits (6). Others (15) have found that short-term hyperoxia was not accompanied by GPX induction in lungs of adult rats. However, it is important to note that we measured selenium-dependent GPX activity in adult murine lung. Jenkinson and coworkers (30) have found that in young rats, lung glutathione S-transferase activities (a reflection of nonselenium-dependent GPX) were increased by hyperoxic conditions similar to ours.

TPUFA measures are considered important for two reasons. First, several investigators consider the expression of vitamin E to TPUFA a meaningful index of tissue antioxidant status (12,14). Only when expressed in this way did lung tissue reflect some influence of both diet and FiO_2 manipulations. Second, losses in TPUFA are often interpreted as indicators of lipid peroxidation in vitro (31). In these studies, dietary treatment influenced plasma TPUFA levels, and not those of lung or any other tissue. Donovan and Menzel (32) also found that dietary vitamin E treatments (0, 10.5 and 105 ppm vitamin E) had no marked effect on murine lung fatty acids. On the other hand, FiO_2 treatment had little impact on plasma, lung or liver TPUFA levels; only adipose TPUFA levels were diminished by hyperoxic exposures. Caution should be advised in interpreting the data for tissue homogenate TPUFA, because these measures usually are performed on intracellular membrane preparations and not on crude tissue homogenates. The dynamic nature of TPUFA levels must be considered; that is, changes in TPUFA may be masked by systemic effects of hyperoxia and dietary vitamin E as well as changes in TPUFA and vitamin E metabolism.

No differences in tissue CD levels were detected among dietary or FiO_2 treatment groups. We selected the CD assay in lieu of the traditional assay for malonaldehyde, because several reports indicated that CD may reflect a greater representation of total peroxidation in vivo (33,34). One problem with this assay is the possibility that compounds other than products of lipid peroxidation may be detected (35). Second, the increased permeability of the lung (lung edema index) and reduced lung cell number (lung DNA) in some mice on hyperoxia may have caused varying degrees of entrapment of CD within the lung vasculature. Third, although the initial stages of lipid peroxidation are accompanied by an accumulation of lipid peroxides and CD, the instability of these products over time may preclude an accurate assessment of the extent of tissue lipid peroxidation (36). Sevanian and coworkers (37) observed that products formed later—malonaldehyde and lipid epoxides—were increased with vitamin E deficiency and NO_2 exposures in newborn rats. Wispe et al. (38) found that expired pentane, and lung thiobarbituric reactants and lipid peroxides were not significantly altered by hyperoxia in newborn rabbits, though IV administration of α-tocopherol reduced the amounts of expired pentane and lung thiobarbituric reactants. On the other hand, lung lipid peroxides were not changed by vitamin E administration. The selection of an appropriate measure for tissue lipid peroxidation remains equivocal. Perhaps, measurement of CD and lipid epoxides along with TPUFA and vitamin E levels in the microsomal fractions may be more meaningful in future experiments, because assays reflecting both early and later stages of lipid peroxidation would be used.

The role of vitamin E as an antioxidant has been examined in a number of experimental animal models in which the degree of lipid peroxidation was inversely related to the tissue level of vitamin E. When expressed in terms of vitamin E to TPUFA ratios, a relatively consistent relationship for specific organs among different species has been noted by several investigators (12–14). Our data are consistent with previous reports in that plasma and lung have the highest amounts of the vitamin relative to TPUFA content (12,14). That these tissues were less susceptible to lipid peroxidation as assessed as CD was not observed in the present study.

In summary, long-term dietary manipulation effected marked changes in lung, adipose and plasma vitamin E levels along with lung GPX activities. Short-term hyperoxia produced changes in plasma TPUFA, adipose TPUFA, lung DNA and, in concert with dietary vitamin E treatments, a modification of vitamin E to TPUFA ratios in lung tissue homogenates of adult female mice. Finally, under the conditions described, plasma vitamin

E levels may be used to predict those in the lung with reasonable confidence.

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REFERENCES

1. Taylor, D.W. (1956) *J. Physiol. 131,* 200-206.
2. Yam, J., and Roberts, R.J. (1979) *Toxicol. Appl. Pharmacol. 47,* 367-375.
3. Gerschman, R., Gilbert, D.L., and Caccamise, D. (1958) *Am. J. Physiol. 192,* 563-571.
4. Hansen, T.N., Hazinski, T.A., and Bland, R.D. (1982) *Pediatr. Res. 16,* 583-587.
5. Bucher, J.R., and Roberts, R.J. (1981) *J. Pediatr. 98,* 806-811.
6. Wender, D.F., Thulin, G.E., Walker Smith, G.J., and Warshaw, J.B. (1981) *Pediatr. Res. 15,* 262-268.
7. Bucher, J.R., and Roberts, R.J. (1982) *Pediatr. Pharmacol. 2,* 1-9.
8. Ward, J.A., and Roberts, R.J. (1984) *Pediatr. Res. 18,* 329-334.
9. McMillan, D.D., and Boyd, G.N. (1980) *Am. Rev. Resp. Dis. 121,* 296-300.
10. Finer, N.N., Peters, K.L., Schindler, R.F., and Grant, G.G. (1981) *Pediatr. Res. 15,* 660-664.
11. Ehrenkrantz, R.A. (1980) *Am. J. Dis. Child. 134,* 1157-1166.
12. Kornbrust, D.J., and Mavis, R.D. (1980) *Lipids 15,* 315-322.
13. Sevanian, A., Hacker, A.D., and Elsayed, N. (1982) *Lipids 17,* 269-277.
14. Poukka Evarts, R., and Bieri, J.G. (1974) *Lipids 9,* 860-864.
15. Yam, J., Frank, L., and Roberts, R.J. (1978) *Pediatr. Res. 12,* 115-118.
16. Tangney, C.C., Aye, P.L., and McCloskey, K.M. (1987) *Nutr. Res. 7,* 265-272.
17. Poland, R.L., Bollinger, R.O., Bozynski, M.E., Karna, P., and Perrin, E.V.D. (1977) *Pediatr. Res. 11,* 577.
18. Tangney, C.C., Driskell, J.D., and McNair, H.M. (1981) *J. Chromatogr. 223,* 389-393.
19. Tangney, C.C. (1984) *J. Liquid Chromatogr. 7,* 2611-2619.
20. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-501.
21. Mason, M.E., and Waller, G.R. (1964) *Anal. Chem. 36,* 583-585.
22. Lawrence, R.A., and Burk, R.F. (1978) *J. Nutr. 108,* 981-987.
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-269.
24. Kissane, J.M., and Robbins, E. (1958) *J. Biol. Chem. 233,* 184-189.
25. Recknagel, R.O., and Ghoshal, A.K. (1966) *Lab. Invest. 15,* 132-147.
26. Till, G.O., Hatherill, J.R., Tourtellotte, W.W., Lutz, M.J., and Ward, P.A. (1985) *Am. J. Pathol. 119,* 376-384.
27. Hull, C.H. and Nie, N.H. (1984) *SPSS Update: New Procedures and Facilities for Release 9,* McGraw Hill Book Co., New York.
28. Norusis, M.J. (1983) *SPSS^X Introductory Statistics Guide,* McGraw Hill Book Co., New York.
29. Chow, C.K., Reddy, K., and Tappel, A.L. (1973) *J. Nutr. 103,* 618-624.
30. Jenkinson, S.G., Lawrence, R.A., Burk, R.F., and Gregory, P.E. (1983) *Toxicol. Appl. Pharmacol. 68,* 399-404.
31. Slater, T.F. (1984) *Methods Enzymol. 105,* 283-293.
32. Donovan, D.H., and Menzel, D.B. (1979) *J. Nutr. 109,* 1856-1864.
33. Buege, J.A., and Aust, S.D. (1978) *Methods Enzymol. 52,* 302-310.
34. Ward, P.A., Till, G.O., Hatherill, J.R., Annesley, T.M., and Kunkel, R.G. (1985) *J. Clin. Invest. 76,* 517-527.
35. Cawood, P., Wickens, D.G., Iversen, S.A., and Dormandy, T.L. (1983) *FEBS Lett. 162,* 239-243.
36. Sevanian, A., and Hochstein, P.)1985) *Ann. Rev. Nutr. 5,* 365-390.
37. Sevanian, A., Elsayed, N., and Hacker, A.D. (1982) *J. Toxicol. Environ. Health 10,* 743-756.
38. Wispe, J.R., Knight, M., and Roberts, R.J. (1986) *Pediatr. Res. 20,* 505-510.

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Trans Fatty Acids. 1. Growth, Fertility, Organ Weights and Nerve Histology and Conduction Velocity in Sows and Offspring

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Effects of dietary *trans* fatty acids on the pre- and postnatal growth and development in pigs were studied with special emphasis on nervous tissue. In experiment 1, female pigs were fed partially hydrogenated fish oil (PHFO) (28% *trans*) or soybean oil (PHSBO) (36% *trans*), in comparison with lard (0% *trans*) from weaning (3 wk) through the first reproduction cycle (up to 2 yr). In experiment 2, female pigs were fed two fish oils (33 and 19% *trans*) in comparison with coconut oil (0% *trans*) in diets with low and high levels of linoleic acid (18:2n-6 *cis,cis*) from gestation until their offspring were three wk old. Compared with the *trans*-free fats, the *trans*-containing fats had no effect on growth and development, feed consumption and utilization or on the weight of the brain, heart, kidneys, liver, lungs or spleen in the adult sows and their offspring. No effects from the experimental fats were found on histology and conduction velocity of the peroneal nerve. An increased number of the sows fed PHFO had fertility problems compared with those fed lard and PHSBO in Expt. 1, but no similar effects were seen in Expt. 2. It is concluded that consumption of *trans* fatty acids with 18–22 carbon atoms from PHFO and with 18 carbon atoms from PHSBO at levels that were 5 to 12 times higher than those normally consumed by humans had no detrimental effects on female pigs or their offspring during pregnancy and lactation.
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The literature on metabolic effects of *trans* fatty acids and their possible nutritional implications is abundant and includes several extensive reviews over recent years (1–8). General biochemical findings include incorporation of *trans* fatty acids into various tissues and fluids of several animal species (9–15), and changes in contents of n-6 polyunsaturated fatty acids (10,14–16) that in some studies (17,18) have been linked to uncoupled phosphorylation. Under extreme experimental conditions, using essential fatty acid (EFA) deficient diets, high intakes of *trans* fatty acids have been found to affect organ weights and cause histological changes (19–21), but no discernible effects on cell or organ function have been found under experimental conditions resembling those of practical human diets (22–27).

Although the nutritional effects of *trans* fatty acids appear to be well-documented in most fields, there are areas in which the knowledge is limited. Thus, the majority of the studies have been conducted with rats, and consequently, there is a need for studies on species that developmentally and metabolically more resemble humans with regard to lipid metabolism, e.g., the domestic pig. Further, long-chain polyunsaturated n-6 and n-3 fatty acids, present in large amounts in nervous tissue (28,29), appear to have a role in nerve functionality, including synaptosomal activity and transmitter uptake (30). EFA deficiency during the period of brain development has caused changes in brain size, and brain lipid class and fatty acid composition (31,32). Experimental data in this field in relation to *trans* fatty acids are, however, meager. Hence, there is a need to explore further the effect of *trans* fatty acids on the composition and functionality of nervous tissue.

Previous studies on *trans* fatty acids have mainly employed partially hydrogenated vegetable oils (PHVO) and, in particular, partially hydrogenated soybean oil (PHSBO), with few experiments on partially hydrogenated fish oil (PHFO). *Trans* fatty acids in PHVO have chain lengths mainly of 18 carbon atoms or less, while PHFO also contain appreciable amounts of *trans* fatty acids with 20 and 22 carbon atoms (33). Thus, there is a need to further elucidate the effects of *trans* fatty acids with 20 and 22 carbon atoms.

It is commonly accepted that *trans* fatty acids in most instances behave differently metabolically from saturated fatty acids (1–8). However, because data on long chain (e.g., 20 and 22 carbon atoms) fatty acids in this regard is rather meager, it is desirable to include saturated fatty acids when long chain *trans* fatty acids are studied.

The main objective of the present series of experiments was to study the nutritional effects of *trans* fatty acids with 20 and 22 carbon atoms in PHFO in comparison with *trans* fatty acids with 18 carbon atoms in PHSBO, and with saturated fatty acids with 20 and 22 carbon atoms in "fully" hydrogenated fish oil (FHFO). Emphasis was placed on nervous tissue. Accordingly, the feeding period comprised the periods of pregnancy and lactation during which most of the nervous tissues are formed. Pigs were chosen as the experimental animal due to their physiological resemblance to man, in particular with regard to brain growth (34–36) and accretion of long-chain polyenoic fatty acids in nervous tissue (28,29).

The study used diets that resemble those of human diets in industrialized countries with regard to fat content and content of EFA, and with varying levels of *trans* fatty acids. The observations comprised overall growth and organ development from three wk until about two yr of age, reproduction, and fetal and early postnatal development of offspring. Development and functionality of nervous tissues were monitored by brain growth and by histology and conduction velocity of the peroneal nerve. The fatty acid composition of brain lipids will be compared with the composition of other organ lipids in later publications.

MATERIAL AND METHODS

The study comprised two experiments. Both experiments used female Norwegian Landrace Pigs obtained from local

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Abbreviations: CF, short chain fatty acids; EFA, essential fatty acids; FHFO, "fully" hydrogenated fish oil/capelin oil; HFO, hydrogenated fish oil; PHFO, partially hydrogenated fish oil; PHSBO, partially hydrogenated soybean oil; PHVO, partially hydrogenated vegetable oil; PUFA, polyunsaturated fatty acids; GLC, gas liquid chromatograph.

breeders. They were offered feed according to practical standards (37) and had free access to water. The pigs were held in environmentally regulated rooms consisting of a grower unit with pens holding five individually fed pigs and a farrowing unit with six individual farrowing crates to which the pigs were moved at day 95 of gestation. The sows were inseminated with stock semen from the same breed. In Expt. 1, 24 three-wk-old piglets were allocated, littermate randomly, to three groups and fed the experimental diets (Table 1). Insemination of the sows was scheduled to achieve equality between groups with regard to age at farrowing and taking into account the capacity of the farrowing unit. Thus, two sows from each group, six sows total, were to be inseminated about every seventh week. Sows that failed to conceive were reinseminated at the next visible oestrus.

In Expt. 2, groups of six six-month-old pigs were allocated randomly to six experimental groups (Table 1) and fed a commercial pig feed until their second oestrus from which time they received the experimental diets. The sows were inseminated at their second visible oestrus.

Sows that did not become pregnant after the first insemination were replaced by a new animal. A total of 37 sows were inseminated to obtain four sows that farrowed on each of the six diets.

Immediately after birth, and without having suckled their mothers, half the males and females in each litter were tested for nerve conduction velocity, and thereafter electrocuted and sampled. The other half were tested for nerve conduction velocity, electrocuted and sampled after three wk of suckling only sow's milk. In Expt. 1, sows were tested for nerve conduction velocity, electrocuted and sampled three wk after parturition. Organs were freed from attached tissues before being weighed.

Diets and experimental fats. The composition of the different diets and the experimental design are shown in Table 1. Thus, Expt. 2 was conducted according to a factorial design with EFA levels as one factor and the experimental fats as the other factor. All diets were isocaloric and isonitrogeneous and contained nutrients to meet the requirements of growing and pregnant pigs (37).

The experimental fats (lard, partially hydrogenated capelin oil = PHFO, melting point 30–32 C, partially hydrogenated soybean oil = PHSBO, melting point 40 C;

TABLE 1

Composition (%) and Contents of Diets

	Experiment 1						Experiment 2					
Basal diets	1 (3-wk to 22 kg body wt.)			2 (22 kg body wt. to termination)			1 (High EFA)			2 (Low EFA)		
Corn starch		12.5										
Ground oat meal		6.0										
Barley meal					11.1			11.3			11.3	
Oat bran meal					19.8			20.0			20.0	
Grass meal					28.7			29.3			32.8	
Soybean meal					26.0			26.3			26.3	
Dried skim milk		74.9			5.8			5.8			5.8	
Sunflower oil		6.0			4.8			3.5			3.8	
Vitamins, minerals, etc.[a]		0.6			3.8			3.8			3.8	
Experimental diets:	A_1	A_2	A_3	B_1	B_2	B_3	1.1	1.2	1.3	2.1	2.2	2.3
Basal diet	84.0	84.0	84.0	86.0	86.0	86.0	86.0	86.0	86.0	86.0	86.0	86.0
Lard	16.0			14.0								
CF							14.0			14.0		
PHFO		16.0			14.0			14.0			14.0	
PHSBO			16.0			14.0						
FHFO									14.0			14.0
Total lipids,[b] wt %,	22.0	22.0	22.0	21.5	21.5	21.5	20.0	20.0	20.0	17.0	17.0	17.0
cal %[c]	41	41	41	41	41	41	39	39	39	35	35	35
Linoleic acid[b], wt %,	3.5	3.5	3.5	4.5	4.3	3.9	2.5	2.9	2.7	0.9	0.9	1.0
cal %[c]	6.6	6.6	6.6	9.2	8.8	7.9	5.1	5.9	5.5	1.9	1.9	2.1
Trans fatty acids, % of total fatty acids,[b]	0.3	20.7	27.8	0.3	18.1	24.3	0.0	23.1	13.7	0.0	28.7	15.3
cal %	0.1	9.1	12.2	0.1	8.0	10.6	0.0	9.5	5.6	0.0	10.4	5.6
P/S ratio[b,d]	0.54	0.98	0.78	0.60	0.85	0.81	0.20	0.47	0.27	0.09	0.19	0.11

[a]Supplement per kg feed: Vit. AD-concentrate 0.2 g, vit. E (Rovimix 25%) 200 mg, thiamine 2 mg, riboflavin 5 mg, niacin 28 mg, pantothenic acid 22 mg, pyridoxine 2 mg, choline-CL (50%) 2 g, vit. B_{12} 0.022 mg, $NaHSeO_3.H_2O$ 0.2 mg, MgO 1.5 g, $ZnCl_2$ 100 mg, $MnSO_4.H_2O$ 30 mg, $FeSO_4.7H_2O$ 200 mg, KI 0.25 mg, $CuSO_4.5H_2O$ 12 mg, NaCl 3 g, $CaPHO_4.2H_2O$ 25.8 g, DL-methionine 0.12 g and ethoxyquin 150 mg.

[b]As analyzed.

[c]Calculated as g protein × 16.75 KJ + g fat × 37.68 KJ + g carbohydrates × 16.75 KJ.

[d]P/F, wt % polyunsaturated fatty acids/wt % saturated fatty acids.

CF, coconut fat; EFA, essential fatty acids, e.g., linoleic acid; FHFO, "fully" hydrogenated fish oil; PHFO, partially hydrogenated fish oil; PHSBO, partially hydrogenated soybean oil.

coconut fat = CF, and "fully" hydrogenated capelin oil = FHFO, melting point 50–52 C) were ordinary human food products of commercial origin. It was intended that the "fully" hydrogenated fish oil should contain only saturated fatty acids. This was only partly achieved. The slight differences in contents of linoleic acids between experimental diets containing similar levels of added sunflower seed oil were due to the content in the experimental fats of linoleic acid or of other fatty acid isomers that overlapped linoleic acid in the gas liquid chromatograph (GLC).

Velocity of nerve conduction. The peroneal nerve, from the hip to the dorsal area, was employed to measure velocity of nerve conduction using an adaption of the method described by Lahoda et al. (38) and an electromyograph model DISA Type 14 A 20 2 Channel with pulses of 50 V strength. The distance between the two electrodes was measured externally and, together with the registered time, used to calculate velocity (m per sec).

Histology of the peroneal nerve. About 5 cm length of the peroneal nerve was dissected out between the hip and the dorsal area and placed in buffered (9.25 g $Na_2PHO_4 \cdot 2H_2O$ + 0.818 $K_2HPO_4 \cdot 2H_2O$ per l) 4% formaldehyde solution. Fixed nerves were cut in longitudinal and transverse sections. After routine embedding in paraffin blocks and sectioning at 5 μ, staining with hematoxylin and eosin and Luxol Fast Blue-cresyl violet (modified Klüver-Banera), microscopic evaluation of nerve, nerve sheet (myelin) and connective tissue was performed. The examinations were carried out without any knowledge of the dietary treatment of the animals.

Chemical methods. Total lipids in the diets were determined as described by Folch et al. (39). An aliquot of the lipids was hydrolyzed in 0.5 N NaOH and methyl esters prepared using BF_3 as a catalyst (40). The fatty acid composition of the lipids was determined by glass capillary GLC (Carlo Erba 2900, on column injector and a Hewlett Packard (3390A) integrator) using a 50-m glass capillary column (CP Sil 88, Chrompack).

Statistical calculations The bulk of the statistical analyses were carried out using simple linear regression programs, plots, and correlation analysis (Minitab statistical package for the micro computer, fundamental version, rd. 82.1). The more complex models (mixed linear models) were analyzed using the PC-ISP statistical package (41). Covariance analyses were applied to separate effects of differences in body wt and ages (sows in Expt. I) from dietary effects on organ weights and nerve conduction velocity.

RESULTS

Expt. 1. Body weights and indices of reproduction in the sows are shown in Table 2. Eight sows were started on

TABLE 2

Growth, Reproduction, Teratogenecity and Nerve Conduction Velocity in Female Pigs[a] and Growth, Body Wt and Nerve Conduction Velocity in Their Offspring (Expt. I)

	Diet 1 (lard)	Diet 2 (PHFO)	Diet 3 (PHSBO)	SEM
No. of animals started	8	8	8	—
No. of animals completed	7	4	4	—
Body wt, kg				
at 21 days (start)	6.2	7.2	6.8	0.5
at 280 days	102.0	102.4	98.9	3.4
at termination	134.4	166.8	147.1	9.2
Feed consumption from 21 days to 280 days, kg	272	272	266	27
Kg feed consumed per kg of body weight gained from 21 to 280 days	2.83	2.86	2.89	0.08
Age at first oestrus, days	231	226	221	5
No. of inseminations to achieve pregnancy	2.5	3.6	2.3	0.8
Length of pregnancy, days	115	115	115	1
Age at parturition, days	467	577	495	44
No. of piglets born per litter	9.6	10.2	10.0	
No. of stillborn piglets per litter	2.7	2.3	0	
Birth wt of piglets, g	1338	1391	1344	32
Body wt of piglets at weaning (3 wk), g	7571	6948	7006	444
Body wt gain from birth to three wk, g	5928	5506	5656	192
Velocity of nerve conduction, m per second:				
Sows	64.7	73.8	63.5	3.6
Newborn piglets	23.2	23.2	24.1	1.6
Three-wk-old piglets	38.0	38.0	39.1	1.4

[a]From three wk of age through their first reproduction cycle.

SEM, standard error of treatment means = (error mean square/number of replicates per treatment)$^{1/2}$.

each diet. Of these, seven sows on Diet 1 (lard), four on Diet 2 (PHFO) and four on Diet 3 (PHSBO) gave birth to sufficient numbers of offspring and completed the experiment. Those eliminated from the experiment were one pig in each of diets 2 and 3 who died of heart failure, one pig on diet 3 that had to be slaughtered due to a mechanical injury, two pigs on Diet 3 who were hermaphrodites, one pig on Diet 2 that did not become pregnant, one pig on Diet 2 that aborted three normal fetuses in the ninth wk of gestation, one pig on Diet 1 that gave birth to only one piglet and one pig on Diet 2 that gave birth to two piglets, one of which was stillborn.

The first sow to become pregnant was bred at an age of 280 days. Growth and development were normal in all pigs and with no significant (not significant means $P > 0.05$) differences between diets for the period 21 to 280 days. Neither were there significant differences between diets in total feed consumption or in the amount of feed consumed per kg gain in body weight. Age at the first visible oestrus and number of oestrus periods (e.g., number of inseminations) required before pregnancy was achieved were not significantly different between diets although, on an average, about one more insemination was needed to achieve pregnancy on Diet 2 compared with Diets 1 and 3. This fact, coupled with practical problems in connection with limited capacity in the farrowing unit, resulted in the sows on Diet 2 being about 100 days older at parturition than those fed Diets 1 and 3. There were no significant differences between diets with regards to length of pregnancy, total number of piglets born or number of stillborn piglets as judged by the Chi-square test. None of the stillborn piglets showed abnormalities on macroscopical examination. There were no significant differences between diets with regard to body weight at birth or growth rate between birth and three wk of age of the piglets (Table 2).

Despite the conduction velocity of the peroneal nerve (Table 2) increasing significantly ($P < 0.05$) from newborn to three-wk-old piglets and sows, neither body weight nor age (in sows) contributed significantly to the variation in the conduction velocity within each age group. There were no significant effects of diets on conduction velocity in the sows or their offspring at birth and at three wk of age. No abnormalities were found on histological examinations of the peroneal nerve and no differences between diets were observed in this respect.

Weights of different organs in sows and offspring are shown in Table 3. There were no significant effects of diets on relative (g per 100 g body weight) weights of brain, heart, kidneys, lungs and spleen in sows and offspring, but sows fed on Diet 2 (PHFO) and 3 (PHSBO) had, on an average, somewhat higher relative liver weights than those fed on Diet 1 (lard).

Expt. 2. Indices of reproduction in sows and body weights and nerve conduction velocity of their offspring at birth and at three wk of age are shown in Table 4. Although the number of sows that were excluded because they did not become pregnant after the first insemination varied between the different diets, the data show no systematic trends with regard to level of linoleic acid or source of experimental fat. There were no significant differences between the different diets with regard to length of pregnancy, number of piglets born or number of stillborn piglets as judged by the Chi-square test and none of the stillborn piglets showed abnormalities on macroscopical examination.

TABLE 3

Relative Weights (g/100 g Body Wt) of Brain, Heart, Kidneys, Liver, Lungs and Spleen of Sows[a] and in Their Offspring (Expt. 1)

Organ	Diet 1 (lard)	Diet 2 (PHFO)	Diet 3 (PHSBO)	SEM
Sows				
Brain	0.06	0.06	0.06	0.00
Heart	0.33	0.31	0.32	0.02
Kidneys	0.22	0.24	0.24	0.03
Liver	1.39	1.65	1.67	0.10
Lungs	0.66	0.71	0.67	0.12
Spleen	0.12	0.10	0.11	0.02
Newborn piglets				
Brain	2.06	2.05	2.10	0.08
Heart	0.62	0.62	0.65	0.03
Kidneys	0.76	0.75	0.78	0.09
Liver	2.77	2.66	2.77	0.29
Lungs	1.51	1.29	1.43	0.13
Spleen	0.09	0.09	0.10	0.01
Three-wk-old piglets				
Brain	0.57	0.64	0.61	0.02
Heart	0.52	0.52	0.49	0.03
Kidneys	0.55	0.56	0.52	0.03
Liver	2.45	2.57	2.44	0.14
Lungs	1.25	1.21	1.38	0.05
Spleen	0.20	0.18	0.19	0.02

[a]From three wk of age and through their first reproduction cycles.
SEM, standard error of treatment means = (error mean square/number of replicates per treatment)$^{1/2}$.

The piglets of sows fed PHFO and FHFO had, on an average, 15% and significantly ($P < 0.005$) lower birth weights compared with those fed CF. This occurred despite the fact that the number of piglets born in these groups was lower than in those fed on CF. There were no significant differences in the birth weight of piglets from sows fed PHFO diets and those on FHFO or between the two levels of dietary linoleic acid. Neither were there significant interactions between levels of linoleic acid and fat source in this respect. Average body weights at three wk are not directly comparable with average birth weights because the latter figures include piglets that were killed after birth. Piglets from sows fed PHFO grew 11% and significantly ($P < 0.05$) faster from birth to three wk than piglets from sows fed CF and FHFO with no significant differences between the latter two groups or between different dietary levels of linoleic acid.

Conduction velocity of the peroneal nerve (Table 4) was significantly ($P < 0.025$) higher in three-wk-old piglets compared with newborn. Within each age group, conduction velocity was significantly ($P < 0.025$) correlated with body weight in newborn ($r = 0.462$), but not in three-wk-old piglets ($P > 0.05$). There were no significant effects of diets on the conduction velocity of the peroneal nerve in newborn piglets. In three-wk-old piglets, offspring from sows fed FHFO had significantly ($P < 0.025$) lower conduction velocity compared with offspring from sows fed CF, while the differences between CF and PHFO, between PHFO and FHFO and between the two levels of dietary

TABLE 4

Reproduction and Teratogenecity in Sows and Body Wt and Nerve Conduction Velocity of Their Offspring (Expt. 2)

Linoleic acid supply:	Low linoleic acid			High linoleic acid			
Experimental fat:	CF	PHFO	HFO	CF	PHFO	HFO	
Diet no.:	1.1	1.2	1.3	2.1	2.2	2.3	SEM
Trans %	0	22.4	13.7	0	28.7	15.3	
No. of animals started	5	7	9	8	4	4	
No. of animals completed	4	4	4	4	4	4	
Length of pregnancy, days	114.0	115.5	115.7	113.7	115.0	114.5	0.9
Age at parturition, days	357	339	342	363	339	369	
No. of piglets born per litter	11.1	11.6	8.6	10.6	10.8	9.0	
No. of stillborn piglets per litter	0.8	1.3	0.3	0.8	0.5	0.5	
Birth weight of piglets, g	1484	1260	1296	1439	1241	1183	66
Body weight of piglets at weaning (3 wk), g	6899	6869	6596	6582	7144	6142	253
Body wt gain from birth to three wk, g	5305	5512	5134	5125	5789	4873	234
Nerve conduction velocity, m per second							
At birth	22.6	21.5	21.3	22.1	22.0	21.9	0.8
At weaning (3 wk)	33.9	34.3	32.3	36.0	33.2	33.2	0.8

SEM, standard error of treatment means = (error mean square/number of replicates per treatment)$^{1/2}$.

TABLE 5

Relative Wt (g/100 g Body Wt) of Brain, Heart, Kidneys, Liver, Lungs and Spleen in Offspring (Expt. 2)

Linoleic acid supply:	Low linoleic acid			High linoleic acid			
Experimental fat:	CF	PHFO	HFO	CF	PHFO	HFO	
Diet no.:	1.1	1.2	1.3	2.1	2.2	2.3	SEM
Newborn							
Brain	1.94	2.29	2.07	1.93	2.22	2.31	0.10
Heart	0.57	0.65	0.61	0.63	0.57	0.64	0.02
Kidneys	0.81	0.86	0.78	0.79	0.76	0.77	0.03
Liver	2.82	2.94	3.10	3.00	2.89	3.04	0.10
Lungs	1.58	1.75	1.35	1.47	1.43	1.29	0.07
Spleen	0.10	0.11	0.10	0.10	0.09	0.09	0.05
Three wk old							
Brain	0.58	0.63	0.63	0.63	0.60	0.70	0.06
Heart	0.49	0.47	0.49	0.49	0.48	0.49	0.01
Kidneys	0.58	0.56	0.61	0.58	0.55	0.56	0.02
Liver	2.61	2.73	2.71	2.74	2.62	2.71	0.04
Lungs	1.31	1.45	1.29	1.43	1.35	1.32	0.06
Spleen	0.20	0.22	0.21	0.22	0.20	0.18	0.10

SEM, standard error of treatment means = (error mean square (number of replicates per treatment)$^{1/2}$.

linoleic acid were insignificant. There was significant ($P < 0.025$) interaction between dietary levels of linoleic acid and CF and PHFO, conduction velocity being higher on high levels compared with low levels of linoleic acid with CF, while the opposite constellation was found with PHFO.

Relative (g per 100 g body weight) brain weight at birth was significantly ($P < 0.05$) higher in offspring from sows on diet 1.2 (PHFO at low level of linoleic acid), compared with those from the other diets.

Brain weights at three wk of age were significantly ($P < 0.005$) higher in offspring from sows fed PHFO and FHFO, compared with those from sows fed CF with no significant differences between the two former groups. Dietary levels of linoleic acid did not affect the brain weights of the offspring (Table 5). Newborn piglets from sows fed low levels of linoleic acid had significantly ($P < 0.01$) higher relative lung weights than newborn piglets from sows fed high levels. Furthermore, piglets from sows fed FHFO had significantly ($P < 0.05$) lower relative lung

weights than piglets from sows fed CF and PHFO that were not significantly different in this respect. There was no significant difference between diets in the relative weights of heart, kidneys, liver and spleen in newborn piglets, but there were significant ($P < 0.05$) interactions between fat source and the level of linoleic acid for relative heart weight. There were no significant effects of diets on the relative weights of brain, heart, kidneys, liver, lungs and spleen in three-wk-old piglets, but there was a significant ($P < 0.05$) interaction between fat source and level of linoleic acid for relative spleen weight.

DISCUSSION

This study used diets with high fat contents, i.e., 35 to 41% of the dietary energy. The contents of linoleic acid varied from about 4 to 1 wt% (about 9-2 cal%) and the P/S (polyunsaturated fatty acids/saturated fatty acids) from about 0.1 to 0.8. The contents of *trans* fatty acids in the experimental diets varied from about 15-30% of total fatty acids (about 6-10% of dietary energy), which provided from about 0.5-1.2 g of *trans* fatty acids per kg of body wt per day. Thus, the experimental diets resembled human diets in industrialized western societies with regard to content of fat and linoleic acid (42), but contained 5 to 12 times more *trans* fatty acids than the estimated average consumption (1).

Dietary *trans* fatty acids could influence nervous tissue directly by being incorporated into neuronal membranes or indirectly by reducing the level of long-chain polyunsaturated fatty acids (PUFA) due to their possible inhibitory effects on the Δ6 and Δ5 desaturases (10,14-16). The outcome of such events could be changes in the conformity and functionality of neuron membranes and synapses due to the altered fatty acid composition. Furthermore, lack of long-chain PUFA could lead to reduced growth and development of neuronal tissue (31,32). We used brain weight and peroneal nerve histology to monitor growth and development and conduction velocity to measure functionality of neuronal tissue. In this series of experiments, significant effects of diets were only seen in three-wk-old piglets in Expt. 2 for brain weight and nerve conduction velocity, and the effects were inconsistent. Despite the fact that the consumption of *trans* fatty acids was high, and for some of the diets combined with a marginal supply of linoleic acid, it did not create adverse effects on nerve tissues as judged by the criteria applied, including nerve histology. It thus appears that very high consumption of *trans* fatty acids, even in combination with a limited supply of linoleic acid over a long period of time and including pregnancy and lactation, do not have negative effects on nerve tissue. To our knowledge, the effects of *trans* fatty acids on brain weight, or histology and conduction velocity of nervous tissue have not been studied.

The limited number of animals to some extent limit the interpretation of the data on the effects of *trans* fatty acids on growth, fertility and teratogenecity or the general toxicological data. However, it is of some concern that fewer of the sows fed the partially hydrogenated fats in Expt. 1 completed the experiment compared with those fed lard. However, heart failure is a common disease in Norwegian landrace pigs, and the fact that one pig in each of the groups fed partially hydrogenated fat died from this disease, while none was affected among those fed lard, may have occurred by chance.

The fact that three out of seven sows on PHFO had major infertility problems (not becoming pregnant, suffering abortion and severely reduced number of piglets) compared with one out of eight on lard (severely reduced number of piglets) and none on PHSBO, appears to be a matter of concern. Further, PHFO-fed sows were, in general, more difficult to get pregnant than the sows fed lard and PHSBO in Expt. 1. However, although a possible relationship to the diet cannot be totally excluded, this explanation is unlikely because the remaining four sows on PHFO that completed the study were equal to or better than the lard-fed sows with regard to their reproduction indices after becoming pregnant. It thus appears that the higher number of PHFO-fed sows with infertility problems compared with those fed lard or PHSBO occurred by chance. This conclusion is partly supported by the results from Expt. 2 where the feeding of PHFO from conception to parturition did not show negative affects on fertility. However, in all groups, pregnancy was more difficult to achieve than is commonly experienced under practical conditions. It is likely that the high fat content of the diets contributed to these results.

The general impression is that dietary *trans* fatty acids with chain lengths of 20 and 22 carbon atoms (PHFO) or 18 carbon atoms (PHSBO) had no consistent effects on growth, reproduction or teratogenecity, when compared with the *trans*-free diets with low content of saturated fatty acids (lard) or high content of short chain fatty acids (CF). Neither were there any differences between PHFO with high content of *trans* fatty acids compared with FHFO containing more saturated long chain fatty acids. These results are in agreement with previous studies with PHVO fed to rats (22,23,27), mice (24) or swine (12), and PHFO fed to swine with adequate levels of EFA (26), and to rats in EFA-deficient diets (20). However, using EFA-deficient diets and EFA-depleted (19) or normal (21) rats, PHVO has been found to decrease growth rate.

The increased lung weights in newborn piglets from sows fed high levels of linoleic acid compared with those fed low levels, and the reduced lung weights in newborn piglets from sows fed FHFO compared with CF and PHFO in Expt. 2 in this study, deserve some comments because Yu and Kinsella (21) observed reduced lung weights in rats fed linolelaidate and partially hydrogenated coconut oil in EFA-deficient diets. However, it is unlikely that the reduced lung weights in our newborn piglets were due to the consumption of *trans* fatty acids, because the content of *trans* fatty acids was lower in FHFO than in PHFO. Furthermore, sows fed PHFO and PHSBO in Expt. 1 had higher liver weights than those fed lard (not significant). Similar observations were made by Vles and Gottenbos (24) using HSBO with 56-65% *trans* fatty acids. However, no histological changes in the livers were found in that study.

Based on the overall results, it may be concluded that consumption of *trans* fatty acids with 18, 20 and 22 carbon atoms from PHFO or with 18 carbon atoms from PHSBO at levels which were 5 to 12 times higher than those normally consumed by humans, had no detrimental effects on female pigs or their offspring, when the diets contained high levels of fat and liberal or limited amounts of linoleic acid.

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REFERENCES

1. Gurr, M.I. (1983) *Doc. Int. Dairy Fed.*, Bull. No. 166, 5-18.
2. Alfin-Slater, R.B. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.) pp. 53-74, American Oil Chemists' Society, Champaign, IL.
3. Emken, E.A. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.) pp. 99-129, American Oil Chemists' Society, Champaign, IL.
4. Kummerow, F.A. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.) pp. 151-179, American Oil Chemists' Society, Champaign, IL.
5. Wood, R. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.) pp. 213-281, American Oil Chemists' Society, Champaign, IL.
6. Kinsella, J.E., Bruckner, J.M., and Shimp, J. (1982) *Am. J. Clin. Nutr. 34,* 2307-2318.
7. Sommerfeld, M. (1983) *Prog. Lipid Res. 22,* 221-233.
8. Emken, E.A. (1984) *Ann. Rev. Nutr. 4,* 339-376.
9. Coots, R.H. (1964) *J. Lipid Res. 5,* 473-476.
10. Hill, E.G., Johnson, S.B., and Holman, R.T. (1979) *J. Nutr. 109,* 1759-1765.
11. Høy, C.-E., and Hølmer, G. (1979) *Lipids 14,* 727-733.
12. Elson, C.E., Benevenga, N.J., Canty, D.J., Grummer, R.H., Lalich, J.J., Porter, J.W., and Johnston, A.E. (1981) *Atherosclerosis 40,* 115-137.
13. Ohlrogge, J.B., Emken, E.A., and Gulley, R.M. (1981) *J. Lipid Res. 22,* 955-960.
14. Hølmer, G., Høy, C.-E., and Kirstein, D. (1982) *Lipids 17,* 585-593.
15. Svensson, L. (1983) *Lipids 18,* 171-178.
16. Thomassen, M.S., Rørtveit, T., Christiansen, E.N., and Norum, K.R. (1984) *Br. J. Nutr. 51,* 315-322.
17. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) *J. Clin. Invest. 34,* 1345-1353.
18. Klausner, R.D., Bhalla, D.K., Dragsten, P., Hoover, R.L., and Karnovsky, M.J. (1980) *Proc. Natl. Acad. Sci. USA 77,* 437-441.
19. Holman, R.T., and Aas-Jørgensen, E. (1856) *Proc. Soc. Exp. Biol. Med. 93,* 175-179.
20. Jensen, B. (1976) *Lipids 11,* 179-188.
21. Yu, P., and Kinsella, J.E. (1980) *Lipids 15,* 975-979.
22. Alfin-Slater, R.B., Wells, A.F., Aftergood, L., and Deuel, H.J., Jr. (1957) *J. Nutr. 63,* 241-261.
23. Nolen, G.A. (1972) *J. Am. Oil Chem. Soc. 49,* 688-693.
24. Vles, R.O., and Gottenbos, J.J. (1972a) *Voeding 33,* 428-433.
25. Vles, R.O., and Gottenbos, J.J. (1972b) *Voeding 33,* 455-465.
26. Svaar, H., Langmark, F.T., Lambertsen, G., and Opstvedt, J. (1980) *Acta Path. Microbiol. Scand. Sect. A, 88,* 41-48.
27. Duthie, I.F., and Barlow, S.M. (1982) in *Nutritional Evaluation of Long-Chain Fatty Acids in Fish Oil* (Barlow, S.M., and Stansby, M.E., eds.) pp. 163-185, Academic Press, London.
28. Purvis, J.M., Clandinin, M.T., and Hacker, R.R. (1982) *Comp. Biochem. Physiol. 72B,* 195-199.
29. Purvis, J.M., Clandinin, M.T., and Hacker, R.R. (1983) *Comp. Biochem. Physiol. 75B,* 199-204.
30. Brenneman, D.E., and Rutledge, C.O. (1979) *Brain Res. 179,* 295-304.
31. Mohrhauer, H., and Holman, R.T. (1963) *J. Neurochem. 10,* 523-530.
32. Galli, C., White, H.B. Jr., and Paoletti, R. (1970) *J. Neurochem. 17,* 347-355.
33. Ackman, R.G. (1982) in *Nutritional Evaluation of Long-Chain Fatty Acids in Fish Oil* (Barlow, S.M., and Stansby, M.E., eds.) pp. 25-88, Academic Press, London.
34. Glanser, E.M. (1966) *Exp. Med. Surg. 24,* 181-190.
35. Book, S.A., and Bustad, L.K. (1974) *J. Anim. Sci. 38,* 997-1002.
36. Dobbing, J., and Sands, J. (1979) *Early Human Dev. 3/1,* 79-83.
37. The National Research Council (1979) in *Nutrient Requirements of Swine,* 8th rev. edn., pp. 52, National Academy of Sciences, Washington, D.C.
38. Lahoda, A., Ross, A., and Issel, W. (1974) in *EMG Primer. A Guide to Practical Electromyography and Electroneurography,* pp. 1-60, Springer-Verlag, Berlin.
39. Folch, Y., Lees, M., and Sloan-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-502.
40. Metcalfe, L.D., Schmitz, A.A., and Pelka, J.R. (1966) *Anal. Chem. 38,* 514-515.
41. The PC-ISP Statistical Package, Champman and Hall Publishing Company, London.
42. Anonymous (1977) *Dietary Fats and Oils in Human Nutrition,* p. 94, Food and Agriculture Organization of the United Nations, Rome.

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Trans Fatty Acids. 2. Fatty Acid Composition of the Brain and Other Organs in the Mature Female Pig

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Female pigs were fed from three wk of age and up to two years a diet containing partially hydrogenated fish oil (PHFO, 28% *trans* monoenoic fatty acids), partially hydrogenated soybean oils (PHSBO, 36% *trans* fatty acids) or lard. No consistent differences were found between PHFO and PHSBO with regard to incorporation of *trans* fatty acids in organ lipids, but *trans* incorporations were highly organ-specific. No *trans* fatty acids were detected in brain phosphatidylethanolamine (PE). The incorporation of monoenoic *trans* isomers, as a percentage of total *cis* + *trans*, in other organs was highest in subcutaneous adipose tissue and liver mitochondria PE, followed by blood lipids with the lowest level in heart PE. The percentage of *trans* isomers compared with that of dietary lipids was consistently lower for 20:1, compared with 18:1 in organs from PHFO-fed pigs. The only effect of dietary *trans* fatty acids on the fatty acid pattern of brain PE was an increased level of 22:5n-6. Heart PE and total serum lipids of pigs fed the hydrogenated fats contained higher levels of 18:2n-6, and these lipids of the PHFO-fed group also contained slightly elevated amounts of 20:3n-6, 18:3n-3 and 20:5n-3. Liver mitochondria PE of the PHFO group also contained higher levels of 20:3n-6 and 22:5n-6. Dietary *trans* fatty acids caused a consistent decrease of saturated fatty acids compensated by increased levels of monoenes. Thus, it may be concluded that dietary long-chain *trans* fatty acids in PHFO behaved similarly metabolically to 18:1-*trans* in PHSBO in pigs, without noticeable influence on brain PE composition and with moderate to slight effects on the fatty acid profile of the other organs.
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Nutritional effects of *trans* isomeric fatty acids are mainly of concern to people in industrialized countries due to their relatively high consumption of partially hydrogenated fats. Thus, the intake of *trans* isomeric fatty acids in these societies was calculated to be about 3–6% of the total fat consumption or about 5–9 g/day per person (1).

Several reports have shown that *trans* isomeric fatty acids are incorporated into membrane lipids of heart (2–4), liver (2–5), brain (5–8) and the developing fetus in rats (7–11), and in several tissues in humans (12). Furthermore, it has been shown that some *trans* isomers of unsaturated fatty acids may affect the tissue content of long chain polyunsaturated fatty acids (LC-PUFA, e.g., 20:4n-6 all *cis*) by reducing the activity of Δ-6 desaturase (13–17), an enzyme that is important in their biosynthesis. Because it has been shown that LC-PUFA is essential for nerve function (18,19), it is particularly important to establish whether consumption of *trans* fatty acids causes incorporation of *trans* fatty acids or alters the LC-PUFA profile in nerve tissue.

Previous studies of *trans* fatty acids have mainly employed partially hydrogenated vegetable oils (PHVO), and in particular partially hydrogenated soybean oils (PHSBO), with few experiments on partially hydrogenated fish oils (PHFO). Because PHFO contains *trans* fatty acids with 20 and 22 carbon atoms, in addition to those with 18 that are prevalent in PHVO (20), there is a need for further studies on PHFO. Furthermore, because most studies on *trans* fatty acids have been conducted with rats, there is a need for data with other animal species that developmentally and metabolically more resemble humans with regard to lipid metabolism, e.g., the domestic pig (21–23).

This study was conducted with pigs to examine the effect of long-term feeding of PHFO in comparison with PHSBO and lard on the incorporation of *trans* fatty acids and the fatty acid profile of the brain PE in comparison with that of other organs.

We have reported the lack of effects of PHFO and PHSBO on growth, histology and conduction velocity of nervous tissue, and on overall growth, teratogenecity and organ weights, while fertility apparently was somewhat impaired on PHFO, but not on PHSBO compared with lard (24).

EXPERIMENTAL

General procedures. Three-wk-old female Norwegian Landrase piglets were allotted to groups of four and fed the experimental diets until three wk after their first parturition as described (24). The diets were isocaloric and isonitrogeneous and composed to meet the nutritional requirement of pigs. Sunflower seed oil (65% linoleic acid) was added to all diets. From three wk of age until the body weight of 22 kg was achieved, the pigs were fed diets containing 5 wt percent sunflower seed oil and 16 wt percent experimental fat. Thereafter, the dietary composition was changed (24), and the content of sunflower seed oil and experimental fat were 4, respectively, 14 wt percent, which together with lipids present in the other feed ingredients gave an analyzed lipid content of about 22%. The experimental fats were lard (control, Diet I), PHFO (partially hydrogenated capelin oil, mp 30–32 C, Diet II) and PHSBO (mp 40 C, Diet III). Fatty acid composition of the diets is shown in Table 1. Diet I had somewhat higher levels of linoleic acid, which evidently was due to linoleic acid present in the lard (6.6%). The fat of the other feed ingredients contained 31% linoleic acid. Agreements in the different diets between analyzed and calculated content of linoleic acid were good, except for the PHFO-diet, in which the analyzed content showed somewhat higher levels than the calculated content. This deviation may have been due to an overlap of unidentified isomers from PHFO and linoleic acid in the gas liquid chromatography.

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Abbreviations: CB, galactocerebrosides; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PHFO, partially hydrogenated fish oils; PHSBO, partially hydrogenated soybean oils; PHVO, partially hydrogenated vegetable oils; PUFA, polyunsaturated fatty acids; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

TABLE 1

Fatty Acid Composition (%) of the Dietary Fats (Total)

	A[a]			B[a]		
	Diet I Lard	Diet II PHFO	Diet III PHSBO	Diet I Lard	Diet II PHFO	Diet III PHSBO
16:0	22.5	11.8	8.8	22.6	16.3	11.2
18:0	15.4	4.0	12.7	14.8	5.6	13.2
20:0	0.3	2.0	0.3	0.3	2.2	0.6
22:0		1.6			2.0	
16:1	1.4	7.0	0.3	1.5	1.1	0.6
18:1-*cis*	33.4	11.6	31.2	34.6	14.1	29.1
18:1-*trans*	0.3	7.0	27.8	0.3	6.8	24.3
20:1-*cis*	0.6	5.6	0.2	0.7	3.9	0.2
20:1-*trans*		7.9			6.0	
22:1-*cis*		6.4			5.7	
22:1-*trans*		5.8			5.3	
18:2n-6	19.9	18.9	16.8	20.8	20.1	18.3
18:3n-3	0.6	0.2	0.2	1.8	2.2	1.9
Others	5.6	10.2	1.7	2.6	8.7	0.6
Sum PUFA	20.5	19.1	17.0	22.6	22.3	20.2
Sum monoenes	35.7	51.3	59.5	37.1	42.9	54.2
Sum saturated	38.2	19.4	21.8	37.7	26.1	25.0
Sum *trans*	0.3	20.7	27.8	0.3	18.1	24.3
P/S[b]	0.54	0.98	0.78	0.60	0.85	0.81

[a]From three wk of age until the body weight of 22 kg (A) and from 22 kg body wt throughout the experiment (B).

[b]Relative amount of PUFA (P) to sum saturated fatty acids (S).

PUFA, polyunsaturated fatty acids.

The pigs were electrocuted about 15 hr after feeding and samples were immediately taken from brain, liver, heart, venous blood (right ventricle) and subcutaneous adipose tissue from the belly.

Chemical methods. Total lipids from samples of blood plasma, heart and adipose tissue were immediately extracted as described by Folch et al. (25). This procedure was modified for the extraction of brain lipids in which the chloroform/methanol ratio was altered from 2:1 to 1:1 (26). Membrane lipids of the liver mitochondria were immediately isolated as described by Hóy and Hólmer (27). All lipid extracts were stored at −25 C, pending further analysis. Lipids of the heart (phosphatidylethanolamines [PE]), the liver mitochondria (PE) and the brain (PE and galactocerebrosides [CB]) were prepared by high performance liquid chromatography (HPLC) from 50 mg samples of the respective lipids, using a Constra Metric III pump with 500 μl loop for injection, and detection by differential refractive index (LDC Mod. 1107). The column (25 × 0.46 cm) material was a cation exchanger (Whatman's Partisil 10 SCX). When isolating CB and PE of the brain, a discontinuous elution (1 ml/min) procedure was used. CB was eluted into two separate fractions, one containing CB of α-hydroxy fatty acids, and the elution times from injection were 2.5 and 3.0 min. CB was eluted by a mixture of 31.3% *n*-propanol, 31.3% chloroform, 31.3% methyl acetate and 6.1% methanol and PE (including PE-plasmalogen) by an eluent composed of 28.1% *n*-propanol, 28.1% chloroform, 28.1% methyl acetate, 11.3% methanol and 4.4% water. Elution time of PE was 2.3 min. When isolating only PE (e.g., heart PE and liver mitochondria PE), the composition of the eluent was 47.0% *n*-propanol, 21.4% chloroform, 21.4% methyl acetate, 6.8% methanol and 3.4% 10 mM acetic acid, and the elution time was 3.5 min. The identity and purity of the isolated lipid classes were verified by thin layer chromatography (TLC) (28) using standard phospholipids (Serdary Research Laboratories Inc., Lond, Ontario, Canada).

The fatty acid composition of the lipids was determined by glass capillary chromatography (GLC) (Carlo Erba 2900, On Column injector and a Hewlett Packard [3390A] integrator) using a 50 m glass capillary column (CP Sil 88 [Chrompack]). Before GLC analysis, the lipids were hydrolyzed by 0.5 N NaOH and methyl esters prepared from methanol using BF_3 as a catalyst (29). *Trans* isomeric double bonds in methyl esters of unsaturated fatty acids were analyzed by IR-spectroscopy (Pye Unicam SP3-200, and 10 μ microcells of sodium chloride). The *trans* content was determined as elaidic acid (18:1n-9 *trans*) equivalents as the percentage of total carbonyl bonds (30).

Statistical methods. The basic principle of the statistical methods used were estimation of the maximum likelihood. The program package chosen was BMDP, program P3V (31).

RESULTS

Brain. The fatty acid composition of the PE in total brain lipids is shown in Table 2. No *trans* fatty acids were detected in any of the dietary groups. Except for 22:5n-6, there were no significant (not significant means $P > 0.05$)

TABLE 2

Fatty Acid Composition (%) in PE of Total Lipids in Brain, Heart and in Liver Mitochondria of Sows[a]

	Brain PE				Heart PE				Liver mitochondria PE			
	Diet I Lard	Diet II PHFO	Diet III PHSBO	Pooled std. dev.[b]	Diet I Lard	Diet II PHFO	Diet III PHSBO	Pooled std. dev.	Diet I Lard	Diet II PHFO	Diet III PHSBO	Pooled std. dev.
16:0	4.9	4.0	4.2	0.7	6.2	5.4	6.0	2.3	10.6	9.1	6.6	4.0
18:0	18.1	15.9	16.3	1.7	23.2	19.9	18.6	3.4	35.3	27.6[c]	24.7[d]	3.3
16:1	0.7	0.9	1.0	0.3	0.7	0.3	0.2	0.5	1.2	1.0	0.9	0.9
18:1-*cis*	23.8	29.6	26.6	3.6	10.8	9.9	14.7	4.2	10.2	12.9	15.8	4.5
18:1-*trans*	—	—	—		0.8	1.7	2.4[b]	0.7	0.3	5.7[d]	8.4[d]	10.0
20:1-*cis*	1.5	2.1	1.9	0.4	0.2	1.0[b]	0.3	0.3	0.1	0.9[d]	0.4	0.3
20:1-*trans*	—	—	—		0.0	0.1[b]	0.0	0.0	0.0	0.3[d]	0.0	0.1
20:3n-9	0.7	0.9	0.8	0.1	0.2	0.2	0.1	0.1	0.1	0.2	0.4[a]	0.2
18:2n-6	0.8	1.7	2.0	0.8	14.3	22.8[c]	19.8	3.6	10.9	11.4	11.9	2.5
20:2n-6	—	—	—		0.3	0.3	0.2	0.2	0.1	0.2	0.2	0.1
20:3n-6	0.9	1.0	1.0	0.1	0.4	0.9[a]	0.5	0.2	0.3	0.8[d]	0.4	0.2
20:4n-6	18.5	17.1	17.6	1.0	33.8	29.5	30.3	7.2	24.4	20.9	24.6	7.3
22:3n-6	0.9	0.7	0.8	0.1	0.1	0.1	0.0	0.1	—	—	—	
22:4n-6	10.1	9.8	9.5	0.5	1.7	1.3	1.1	1.2	0.5	1.3	0.5	0.8
22:5n-6	0.9	1.4[a]	1.3	0.3	0.2	0.6	0.2	0.2	0.1	0.4[a]	0.2	0.2
18:3n-3	0.2	0.1	0.1	0.8	0.2	0.4[a]	0.4	0.1	0.2	0.3	0.3	0.2
20:5n-3	—	—	—		1.1	2.1[c]	1.4	0.4	0.8	0.9	0.6	0.7
22:5n-3	1.0	0.8	0.8	0.3	2.4	2.0	2.3	0.7	1.7	2.0	2.4	0.9
22:6n-3	16.5	14.8	15.2	2.2	3.0	2.1	1.6	1.5	3.4	4.1	2.0	1.1
Sum PUFA n-6	32.1	31.7	32.2		50.8	55.5	52.1		36.3	35.0	37.8	
Sum PUFA n-3	17.7	15.7	16.1		6.7	6.6	5.7		6.1	7.3	5.3	
Sum PUFA total	50.5	48.3	49.1		57.7	62.3	57.9		42.5	42.5	43.5	
Sum monoenes	26.0	32.6	29.5		12.5	12.9	17.6		11.7	20.8	25.5	
Sum saturated	23.0	19.9	20.5		29.4	25.3	24.6		45.9	36.7	31.3	
Sum *trans*	0	0	0		0.8	1.8	2.4		0.3	6.0	8.4	

[a]Fed these diets from three wk of age through their first reproduction cycle (up to 2 years).

[b]Pooled std. dev. = pooled standard deviation, (error mean square)$^{1/2}$.

Means that deviate significantly from the corresponding values of the control diet (diet I) are shown by superscript a ($P < 0.05$), b ($P < 0.025$), c ($P < 0.01$) and d ($P < 0.005$). Average of four replicate determinations.

differences in fatty acid composition between the different groups. The relative portion of 22:5n-6 in the respective groups fed PHFO and PHSBO were about 55% ($P < 0.05$) and 45% ($P < 0.05$) higher than that in the lard-fed group.

Heart. Fatty acid composition of the PE in total heart lipids is shown in Table 2. PE from the heart of sows fed PHFO and PHSBO contained appreciable levels of 18:1-*trans*, but only traces of 20:1-*trans*. However, noticeable levels of 18:1-*trans* were detected in heart PE from sows fed lard.

Feeding PHFO caused a significant ($P < 0.01$) increase in the percentage of linoleic acid, and a somewhat less but significant ($P < 0.05$) increase of 20:3n-6, compared with lard feeding. Feeding PHSBO, the same effects as shown for the PHFO-fed group were found, although the variations were insignificant compared with the lard-fed group. Further, feeding of any one of the hydrogenated fats, in particular PHFO, caused a significant increase in the percentages of 18:3n-3 ($P < 0.05$) and 20:5n-3 ($P < 0.01$), with compensatory but smaller, insignificant reductions in the percentages of 22:5n-3 and 22:6n-3. The different dietary fats led to variations in the sum of the different fatty acid classes. Thus, compared with lard, feeding PHFO increased the percentage of PUFA n-6, had no effect on the percentage of PUFA n-3 or monoenoic fatty acids and decreased the percentage of saturated fatty acids. PHSBO-feeding, on the other hand, led to reduced percentages of PUFA n-3 and saturated fatty acids with a compensatory increase in the percentage of PUFA n-6 and monoenoic fatty acids compared with lard feeding.

Liver. The fatty acid composition of the PE in the liver mitochondria is shown in Table 2. As was found in heart PE, *trans* isomers of 18:1 and *cis* and *trans* isomers of 20:1 were present also in liver mitochondria PE and were significantly influenced by the dietary treatment ($P < 0.005$). Feeding PHFO, and in particular PHSBO, the percentages of stearic acid were significantly ($P < 0.005$) reduced compared with lard. Feeding with PHFO, but not with PHSBO, increased the percentages of 20:3n-6 ($P < 0.005$) and 22:5n-6 ($P < 0.05$). The overall effect of feeding PHFO and PHSBO, compared with lard feeding, was an increase in the relative portion of monoenoic fatty acids

TABLE 3

Fatty Acid Composition (%) of Total Lipids of Plasma and of Subcutaneous Adipose Tissue in Sows[a]

	Plasma				Subcutaneous adipose tissue			
	Diet I Lard	Diet II PHFO	Diet III PHSBO	Pooled std. dev.	Diet I Lard	Diet II PHFO	Diet III PHSBO	Pooled std. dev.
16:0	20.0	15.6[d]	11.4[d]	1.4	21.7	20.8	12.3[d]	1.3
18:0	13.4	9.9[d]	9.8[d]	1.3	10.1	6.4[d]	7.6	1.3
16:1	1.5	2.9[c]	1.0	0.6	2.0	4.2[d]	1.3[b]	0.7
18:1-*cis*	23.9	16.8[c]	25.0	3.1	39.3	21.7[d]	39.3	3.8
18:1-*trans*	0.0	4.3	7.1[b]	3.2	0.0	5.7[a]	13.4[d]	3.5
20:1-*cis*	0.2	1.7[d]	0.2	0.5	1.4	5.5[d]	0.9	0.8
20:1-*trans*	—	—	—		0.0	6.2[d]	0.3	0.9
22:1-*cis* + *trans*	0.0	0.9[d]	0.0	0.2	trace	3.0[d]	0.2	0.5
20:3n-9	—	—	—		0.9	0.9	0.6[a]	0.4
18:2n-6	29.8	38.7[d]	34.5[b]	2.4	22.2	23.0	21.5	1.3
20:2n-6	0.3	0.2	0.0	0.2	—	—	—	
20:3n-6	0.2	0.4[b]	0.3	0.1	0.3	0.2	0.1	0.1
20:4n-6	7.2	5.0	6.3	1.7	0.5	0.8	0.5	0.4
22:4n-6	—	—	—		0.3	0.0[d]	0.1[d]	0.1
18:3n-3	1.4	1.8[a]	1.6	0.3	1.3	1.4	1.4	0.2
20:5n-3	0.3	0.9[b]	0.5	0.3	—	—	—	
22:5n-3	0.6	0.6	0.8	0.3	0.3	0.0[d]	0.1	0.1
22:6n-3	1.0	0.2	0.9	0.6	0.2	0.0[b]	trace	0.1
Sum PUFA n-6	37.5	44.3	41.1		23.3	24.0	22.2	
Sum PUFA n-3	3.3	3.5	3.8		1.8	1.4	1.5	
Sum PUFA total	40.8	47.8	44.9		26.0	26.3	24.3	
Sum monoenes	25.6	26.6	33.3		42.7	46.3	55.4	
Sum saturated	33.4	25.5	21.2		31.8	27.2	19.9	
Sum *trans*	0	4.3	7.1		0	11.9	13.4	

[a]Fed these diets from three wk of age through their first reproduction cycle (up to 2 years).

Pooled std. dev. = pooled standard deviation, (error mean square)$^{1/2}$.

Means that deviate significantly from the corresponding values of the control diet (diet I) are shown by superscript a ($P < 0.05$), b ($P < 0.025$), c ($P < 0.01$) and d ($P < 0.005$). Average of four replicates.

and a decrease in saturated fatty acids with negligible effects on the percentages of n-6 and n-3 PUFA.

Blood. The fatty acid composition of venous blood lipids (total) is shown in Table 3. The dietary fats had a profound effect on the fatty acid composition of the blood lipids.

Total plasma lipids from sows fed hydrogenated fats, in particular PHSBO, contained considerable amounts of 18:1-*trans* isomers. Long-chain *trans* isomers (i.e., 20:1-*trans*) were not detected in blood lipids, and the percentages of the *cis* isomeric homologs were low. Due to the low percentages of 22:1 isomers, we were not able to analyze separately for *cis* and *trans* isomers. The dietary level of *trans* 22:1 was about 50% of the total content of 22:1. Considering the dietary composition and the exclusion of 20:1-*trans* from the blood lipids, it seems reasonable to assume that, at most, 50% of 22:1 in blood lipids were *trans* isomers. No long-chain saturated fatty acids (e.g., 20:0, 22:0 and 24:0) were detected. Compared with those of sows fed lard, blood lipids of sows fed PHFO and PHBO contained more linoleic acid and less arachidonic acid, only the first different being significant ($P < 0.025$). Other n-6 fatty acids were not noteworthily different between the groups, except for 20:3n-6, which was increased on PHFO ($P < 0.025$) and PHSBO ($P > 0.05$). Feeding with PHFO caused a significant increase in the percentages of 18:3n-3 ($P < 0.05$) and 20:5n-3 ($P < 0.025$). Sows fed the hydrogenated fats had significantly ($P < 0.005$) lower relative portions of 16:0 and 18:0, compared with the lard-fed group. Sows fed PHFO had significantly higher percentage of 16:1 ($P < 0.01$) and lower percentage of 18:1 *cis* ($P < 0.01$), compared with sows fed PHSBO and lard. In general, feeding with partially hydrogenated fats increased the relative portion of PUFA n-6 and also, to some extent, that of monoenoic fatty acids, and reduced the percentage of saturated fatty acids in the blood lipids, compared with lard feeding.

Adipose tissues. The fatty acid composition of subcutaneous adipose tissue from the belly fat is shown in Table 3. Significant percentages of 18:1-*trans* isomers were found in sows fed PHSBO ($P < 0.005$) and of 18:1-*trans* ($P < 0.05$) and 20:1-*trans* ($P < 0.005$) in sows

fed PHFO. The percentage of 22:1-*trans* isomers could not be determined and was calculated as described for the blood lipids.

Also, appreciable percentages of long-chain monoenoic (20:1 and 22:1) *cis* fatty acids, but not their saturated homologs, were found in sows fed PHFO. The relative portions of linoleic and arachidonic acids were not significantly different between groups. However, PHFO led to significant reduction in the percentage of 22:4n-6 ($P < 0.005$), 22:5n-3 ($P < 0.005$) and 22:6n-3 ($P < 0.025$), and PHSBO, a reduction in the percentage of 22:4n-6 ($P < 0.005$), compared with lard. In general, feeding of partially hydrogenated fats increased the percentages of monoenoic fatty acids and reduced the percentages of saturated fatty acids, but had minor effects on the percentage of PUFA, compared with lard feeding.

DISCUSSION

This study used diets with contents of fat and linoleic acid resembling those of human diets in industrialized countries but containing five times more *trans* fatty acids (i.e., 25% vs 5% of total fatty acids) (1). Further, pigs were used as experimental animals due to their resemblance to humans (20–22), and the study comprised a noticeable part of their life-span period, i.e., about two years. This paper presents data with the aim of exploring if isomeric fatty acids are incorporated into nervous tissue or if the presence of *trans* fatty acids in the diet affects the metabolism of fatty acid in such a way that the overall fatty acid profile and, in particular, the relative portion of PUFA is changed. The emphasis was on nerve tissue (i.e., brain), but it was felt necessary to compare reactions in the brain with those of structural (i.e., heart and liver mitochondria) and non-structural (i.e., blood and adipose tissue) lipids in other organs. Analyzing other organs than the brain also facilitated comparison of results from this study with those of other studies not involving nervous tissue (2–5). Phosphatidylcholine (PC) and PE are, in general, the main structural lipids of the cell membranes. Brain phospholipids of different species contain about 30–38% PC and 30–42% PE (32).

Regretfully, our capacity did not permit us to analyze both PC and PE. Due to the fatty acid composition, i.e., the high content of long-chain polyunsaturated fatty acids (LC PUFA) in PE, compared with that of PC (33), and the assumed structural importance of these LC PUFA, the fatty acid composition of PE was chosen as an indicator for the metabolic effect of *trans* fatty acids. Also, PE was chosen to facilitate the comparison of our results with those of other investigations (5,33,34).

The identification of fatty acid isomers in partially hydrogenated fats is a tedious and difficult task. In particular, this is the case for PHFO containing positional and geometrical isomers of the LC PUFA abundant in marine lipids (e.g., 18:4n-3, 20:5n-3 and 22:6n-3) (20). Our inability to identify the numerous fatty acid isomers led us to search for an alternative method to determine *trans* fatty acids, i.e., IR-spectrophotometry. However, *trans* isomerism analyzed by IR-spectrophotometry showed higher values of total *trans* content (1.5–4 times) than the sum of identified *trans* fatty acids determined by GLC. This was found for all lipid extracts analyzed, except for the brain CB, in which only IR-measurements were performed, and was particularly pronounced for organ and dietary lipids related to PHFO. The differences may partly be due to the fact that some *trans* isomers were not identified by the GLC-analysis. Our results are in line with those of Perkins et al. (35), who compared the content of *trans* fatty acids determined by GLC (packed columns) analysis with total content of *trans* isomerism analyzed by IR-spectrophotometry in margarine. However, the deviation between GLC and IR-analysis in our experiment was greater than would be expected from their results. The molar IR-absorption of fatty acid *trans* isomers might depend on the chain lengths, the position of the *trans* double bond in the chain, the degree of unsaturation (i.e., monoenoic vs polyenoic fatty acids) and the plurality of *trans* double bonds in the chain (36).

In our analysis, the total *trans* content was calculated as percent methyl elaidate (18:1n-9 *trans*) equivalents, i.e., the IR absorption of the fatty acid methyl esters of the samples compared with that of the methyl elaidate. This may have led to incorrect estimates of the total *trans* contents. Admitting that there may be sources of error in both GLC and IR determinations of *trans* isomerism, we decided to use the GLC analysis as the most accurate one.

Ohlrogge et al. (12), studying the distribution of *trans* fatty acids in human organ lipids, also detected *trans* fatty acids in brain lipids. Brain lipids of rats fed *trans* fatty acids also were reported to contain *trans* fatty acids (5–8). In this study, no *trans* fatty acids were detected in brain PE. Similar results were obtained by analyzing the total lipids extracted from brain (not reported) and from the IR-spectrophotometric analysis of brain CB (results not reported). This was in contrast with other structural lipids such as heart and liver mitochondria PE, in which significant amounts of *trans* 18:1 and 20:1 were found. It should be noted that lipids of liver mitochondria and, in particular, of heart from lard-fed pigs also contained 18:1 *trans*. These results are in line with that reported by Wood (33), who examined various tissues from rats maintained on a chow diet, and by Royce et al. (37), who found that the total heart lipids of weanling pigs fed lard for six months contained about 0.6% 18:1-*trans*. Although our control diet did contain low levels of *trans* 18:1, this cannot explain the presence of *trans* fatty acids found in the PE of heart and liver mitochondria, particular since *trans* fatty acids were not detected in blood lipids and adipose tissue. The source of the *trans* fatty acids in PE of heart and liver mitochondria, therefore, are obscure.

The difference between different organs and the effect of fatty acid chain length of the monoenes (i.e., 18 and 20 carbon atoms) and fat sources (PHFO and PHSBO) with regard to incorporation of *trans* fatty acids may be judged from the discrimination factor (e.g., the relation of the percentage *trans* isomers of the total monoene content in the organ lipids to that of the respective dietary lipids) as shown in Table 4. Thus, there was apparently little discrimination against 18:1-*trans* fatty acid incorporation into the liver mitochondria PE, while the level of *trans* fatty acids in heart PE and blood was somewhat reduced, compared with the diet. There was no consistent difference in the incorporation of 18:1-*trans* from PHFO and PHSBO. The incorporation of 20:1-*trans* was consistently lower than that of 18:1-*trans*. It is of interest that the content of 20:1-*trans* in the organ lipids, relative the content

TABLE 4

Ratio of the Percentage *trans* Isomers of the Total Monoene Content of Organ Lipids to That of the Respective Dietary Fats

	Tissues				
	Brain	Heart	Liver	Blood	Adipose
18:1-*trans* PHFO	0	0.45	0.94	0.63	1.33
PHSBO	0	0.32	1.22	0.51	0.58
20:1-*trans* PHFO	0	0.15	0.41	0	0.87

of the dietary lipids, showed a similar picture as that for 20:1-*cis* and 22:1 *cis* plus *trans*, i.e., a significant discrimination against the dietary long-chain monoenoic acids irrespective of the geometry of the double bond. The analysis of total *trans* isomerism determined by IR-spectrophotometry (results not shown) support the conclusion on the organ specific incorporation of *trans* fatty acids revealed by the GLC analysis, i.e., no *trans* isomerism due to dietary treatment was detected in brain PE and brain CB.

Previous studies have shown somewhat elevated levels of linoleic acid and reduced levels of arachidonic acid in tissue lipids of animals consuming *trans* fatty acids (38). These findings have been explained by that *trans* fatty acids may interfere with the biosynthesis of arachidonic acid (20:4n-6) from linoleic acid (18:2n-6). One explanation postulated is that 18:1-*trans* fatty acids exert a competitive inhibition on the activity of the enzyme Δ-6 desaturase (13,39,40), while *trans* isomers of 20:1 and 22:1 fatty acids have been found to inhibit both Δ-6 and Δ-5 desaturases (16,17). In our study, the feeding of *trans* fatty acids and, in particular, PHFO increased the relative portion of linoleic acid in total plasma lipids and heart PE.

However, despite the fact that the percentages of arachidonic acid and of the other long-chain n-6 fatty acids were slightly reduced in those organ lipids, the reduction did not compensate for the increased percentages of linoleic acid. It is worth noting that the percentages of total PUFA n-6 in blood and heart lipids were higher in the groups fed hydrogenated fats, compared with that of the lard-fed group. Furthermore, liver mitochondria PE, heart PE and the lipids of venous blood of pigs fed PHFO, but not PHSBO, contained significantly higher levels of 20:3n-6, compared to that of the lard-fed groups. These results may support the hypothesis on inhibition of the enzymatic activity of Δ-5 desaturase by *trans* isomers of 20:1 and 22:1 fatty acids in PHFO (16,17).

The only effect of dietary *trans* fatty acids on brain PE in our study was an increased level of 22:5n-6 in PHFO-fed pigs. A similar observation was made by Sanders et al. (34) in the brain PE of rats fed margarine. Because the brain did not contain detectable amounts of *trans* fatty acids in our study, it is possible that the increased percentage of 22:5n-6 was a consequence of the fatty acid metabolism in organs outside the brain (e.g., the liver). Increased percentage of 22:5n-6 was, except for the brain, only found in PE of the liver mitochondria of the PHFO-fed pigs. This coordinated effect of dietary PHFO on the fatty acid pattern of liver mitochondria-PE and brain-PE may be explained by the appearance of long-chain *trans* isomeric fatty acids and the biochemical activity in these organs, i.e., the capability to synthesize LC PUFA (41).

It thus may be hypothesized that dietary *trans* fatty acids in the liver partly inhibited Δ-6 desaturases and Δ-5 desaturases and, consequently, elevated levels of 18:2n-6 and 20:3n-6 were transported to the brain. Due to the elongation/desaturation capacity of the brain (41), those substrates (e.g., 18:2n-6 and 20:3n-6) were metabolically transformed to 22:5n-6.

Feeding PHFO led to increased levels of linolenic acid (18:3n-3) and 20:5n-3 in heart PE and the blood lipids. This may be explained by a relative inhibition of the desaturation/elongation of n-6 fatty acids by the LC *trans* fatty acids that favored the biosynthesis of 20:5n-3.

Because it has been discussed (42) whether *trans* monoenoic fatty acids resemble *cis* monoenoic or saturated fatty acids in their metabolism, a closer examination of the effect of PHFO and PHSBO on the percentage of the different fatty acid classes (i.e., PUFA, *cis*, respectively, *trans* monoenoic and saturated fatty acids) in the respective organs may be relevant. Whenever the feeding of partially hydrogenated fats affected the level of fatty acid classes in an organ, there was a striking similarity in the effect of PHFO and PHSBO. In all organs studied, the total levels monoenoic fatty acids were changed in the same direction and opposite to that seen for saturated fatty acids. This influence increased as the percentage of *trans* fatty acids in the organ lipids increased and was most striking for PHSBO-fed animals. It might be possible to conclude from these observations that the *trans* monoenoic fatty acids simply replaced the saturated fatty acids in the lipid molecules. An equally justified explanation would be that *trans* and *cis* monoenes follow the same metabolic pathway.

Based on the combined results, it may be concluded that PHFO and PHSBO show similar metabolic effects on fatty acid composition in the organs of pigs after long feeding. None of them affect the fatty acid profile of the brain-PE to a significant degree, while small amounts of *trans* fatty acids are incorporated into other organs and slight changes in the fatty acid profile may be observed. These results confirm and extend previously reported lack of adverse effect from *trans* fatty acids on nerve conduction velocity and general biological performance (24).

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REFERENCES

1. Emken, E. (1984) *Ann. Rev. Nutr. 4,* 339–376.
2. Reichwald-Hacker, I., Grosse-Oetringshaus, S., Kiewitt, I., and Mukherjee, K.D. (1980) *J. Nutr. 110,* 1122–1129.
3. Reichwald-Hacker, I., Ilsemann, K., and Mukherjee, K.D. (1979) *J. Nutr. 109,* 1051–1056.
4. Moore, C.E., Alfin-Slater, R.B., and Aftergood, L. (1980) *Am. J. Clin. Nutr. 33,* 2318–2323.
5. Lawson, L.D., Hill, E.G., and Holman, R.T. (1983) *J. Nutr. 113,* 1827–1835.
6. Cook, C.W. (1978) *J. Neurochem. 32,* 515–519.
7. Menon, K.M., and Dhopeshwarkar, G.A. (1982) *Prog. Lipid Res. 21,* 309–326.

8. Karney, R.I., and Dhopeshwarkar, G.A. (1978) *Biochim. Biophys. Acta 531,* 9-15.
9. Moore, C.E., and Dhopeshwarkar, G.A. (1980) *Lipids 15,* 1023-1028.
10. Menon, N.K., and Dhopeshwarkar, G.A. (1982) *Fed. Am. Soc. Exp. Biol. 41,* 626.
11. Ono, K., and Fredrickson, D.S. (1964) *J. Biol. Chem. 239,* 2482-2488.
12. Ohlrogge, J.B., Emken, E.A., and Gulley, R.M. (1981) *J. Lipid Res. 22,* 955-960.
13. Kurata, N., and Privett, O.S. (1980) *Lipids 15,* 1029-1036.
14. Yu, P., Mai, J., and Kinsella, J.E. (1980) *Lipids 15,* 975-979.
15. Mahfouz, M.M., Johnson, S., and Holman, R.T. (1980) *Lipids 15,* 100-107.
16. Svensson, L. (1983) *Lipids 18,* 171-178.
17. Kirstein, D., Høy, C.-E., and Hølmer, G. (1983) *Br. J. Nutr. 50,* 749-756.
18. Crawford, M.A. (1980) *Prog. Food Nutr. Sci. 4,* 75-80.
19. Sinclair, A.J. (1975) *Proc. Nutr. Soc. 34,* 287-291.
20. Ackman, R.G. (1982) in *Nutritional Evaluation of Long Chain Fatty Acids in Fish Oil* (Barlow, S.M., and Standsby, M.E., eds.) pp. 25-89, Academic Press Inc., London.
21. Dobbin, J., and Sands, J. (1979) *Early Human Dev. 3,* 79-83.
22. Glauser, E.M. (1966) *Exp. Med. Surg. 24,* 181-190.
23. Purvis, J.M., Clandinin, M.T., and Hacker, R.R. (1982) *Comp. Biochem. Physiol. 72B,* 195-199.
24. Opstvedt, J., and Pettersen, J. (1988) *Lipids 23,* 714-720.
25. Folch, J., Lees, M., and Sloan-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-502.
26. Phillipis, F., and Privett, O.S. (1979) *Lipids 14,* 590-595.
27. Høy, C.-E., and Hølmer, G. (1979) *Lipids 14,* 727-733.
28. Vitiello, F., and Zanetta, J.-P. (1978) *J. Chromatogr. 166,* 637-640.
29. Metcalfe, L.D., Schmitz, A.A., and Pelka, J.R. (1966) *Anal. Chem. 38,* 514-515.
30. Hølmer, G., and Aas-Jørgensen, E. (1969) *Lipids 4,* 507-514.
31. "BMDP Statistical Software" Program: General Mixed Model Analysis of Variance BMDP 3V (1983) University of California Printing and Edition Press.
32. Sastry, P.S. (1985) *Prog. Lipid Res. 24,* 69-176.
33. Wood, R. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.) pp. 213-281, American Oil Chemists' Society, Champaign, IL.
34. Sanders, T.A.B., Mistry, M., and Naismith, D.J. (1984) *Br. J. Nutr. 51,* 57-66.
35. Perkins, E.G., McCarthy, T.P., O'Brien, M.A., and Kummerow, F.A. (1977) *J. Am. Oil Chem. Soc. 54,* 279-281.
36. Scholfield, C.R. (1979) in *Geometrical and Positional Fatty Acid Isomers* (Emken, E.A., and Dutton, H.J., eds.) pp. 17-52, American Oil Chemists' Society, Champaign, IL.
37. Royce, M.S., Holmes, R.P., Takagi, T., and Kummerow, F.A. (1984) *Am. J. Clin. Nutr. 39,* 215-222.
38. Rønneberg, R., and Lambertsen, G. (1986) *Fiskeridir. Skr. - Ernæing 2,* 201-211.
39. De Schrijver, R., and Privett, O.S. (1982) *J. Nutr. 112,* 619-626.
40. Mahfouz, M.M., Smith, T.L., and Kummerow, F.A. (1984) *Lipids 19,* 214-222.
41. Naughton, J.M. (1981) *Int. J. Biochem. 13,* 21-32.
42. Gurr, M.J. (1983) *Doc. Int. Dairy Fed.,* Bull. No. 166, 5-18.

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Lipids from the Paracloacal Glands of the American Alligator *(Alligator mississippiensis)*

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Lipids from the paracloacal glands of adult and of immature American alligators *(Alligator mississippiensis)* were analyzed by gas chromatography-mass spectrometry. Acetate esters (C_{12}–C_{18}) were indicated in the adults' secretions. Immature alligators contain C_{10}–C_{18} acetates, C_{12}–C_{18} dodecanoates, tetradecanoates and other high molecular weight esters, and C_{10}–C_{16} 3-methylbutanoates. Cholesterol, C_{16} and C_{18} free fatty acids, and α-tocopherol (vitamin E) were detected in some samples. The results are compared with those published for South American caiman species.
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All extant crocodilians possess paired exocrine organs known as paracloacal glands that discharge secretions through duct openings in the lateral walls of the cloaca. A histological study of the American alligator *(Alligator mississippiensis)* indicated that each gland is a single secretory sac with a single duct (1). Parenchymal cells containing lipid droplets migrate toward and accumulate in a cylindrical conglomerate that partially occludes a lumen in the center of the gland. Secretions from degenerating cells of the central conglomerate collect near the secretory duct. A histochemical test indicated lipids in the glandular exudate.

Fester and Bertuzzi (2) analyzed chemicals from the paracloacal glands of two caimans, *Alligator sclerops* (= *Caiman crocodilus*) and *A. latirostris* (= *C. latirostris*), where secretions apparently were pooled from both species. Fatty acids, nitrogenous bases, glycerol, cholesterol and traces of phosphorus-containing compounds were reported among the constituents. A nine-carbon alcohol dubbed "yacarol" also was indicated. The melting point, refractive index and other physical properties of this constituent were taken to indicate *d*-citronellol (3). This compound remained unchanged following years of storage in an inert atmosphere (4).

We report here on the identification of lipids present in the paracloacal glands of adult and of immature *A. mississippiensis.* This is the first detailed description of paracloacal gland chemicals from this species.

METHODS

Secretions were obtained from 80 immature (total lengths = 45–118 cm) and five adult (1.5–3.0 m) alligators from Port Arthur, TX (Murphree Wildlife Refuge) and 15 adults (1.6–3.3 m) from Grand Chenier, LA (Rockefeller Wildlife Center) during September 1985. Secretions from immature (unsexed) and adult alligators were kept separate. Materials were pooled according to sex for eight males and seven females from Louisiana. The adult alligators were sampled within one hr after their death. Samples from living, immature alligators were collected after they had been hand-captured and confined with 15 or more individuals for several hours.

Paracloacal gland secretions were collected by manually everting the glands and squeezing their contents into glass vials containing methylene chloride (CH_2Cl_2). The vials were wrapped individually in paper towels and shipped on dry ice. They were kept frozen (−4 C) before extraction with CH_2Cl_2 and before analysis of CH_2Cl_2-soluble material.

The CH_2Cl_2 extracts were drawn off with pipettes and analyzed by capillary gas chromatography-mass spectrometry (GC-MS) using either a 30 m × 0.32 mm SE-54 column (0.25 μm) or a 30 m × 0.32 mm SPB-5 column (0.25 μm) connected to a Finnigan 4500B mass spectrometer. Spectra and retention times were compared with those of authentic compounds, obtained commercially, or in the case of esters, prepared from the appropriate acid chloride and alcohol. The mass spectra of the 3-methylbutanoates and the isomeric pentanoates differed significantly from one another.

RESULTS AND DISCUSSION

Hexadecyl acetate (Fig. 1) was a major constituent in most samples of alligator paracloacal gland secretions (Table 1). In addition, two other C_{16} acetates were detected; one contained one double bond, the other, two double bonds (positions unknown). Tetradecyl acetate, dodecyl acetate, and relatively small quantities of octadecenyl and octadecadienyl acetates (double bond positions unknown) also were present in many adult samples.

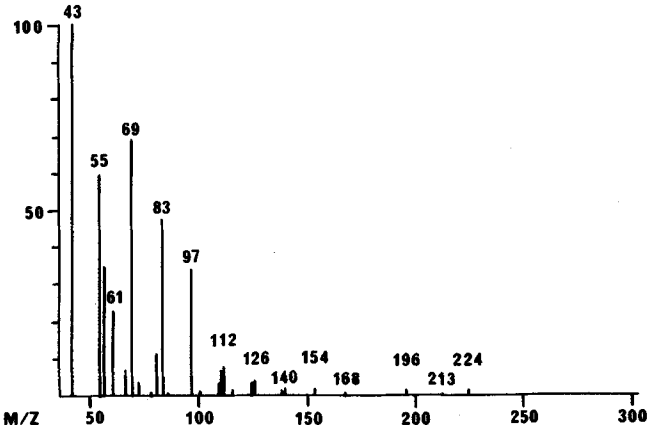

FIG. 1. Mass spectrum of hexadecyl acetate. m/z 43 (CH_3CO^+), 61 ($CH_3CO_2H_2^+$), 224 ($C_{16}H_{32}^+$).

*To whom correspondence should be addressed.
Abbreviations: GC-MS, gas chromatography-mass spectrometry.

TABLE 1

Lipids Indicated as Major (maj, ≥25%), Minor (min, <20%) and Trace (tr, <5%) Constituents of Alligator Paracloacal Glands

		Sample source and identity, with number of individuals in each sample										
		Louisiana					Texas					
		Adult					Adult		Immature			
Compound	m.w.	1♂	1♂	6♂	3♀	4♀	3♂	1♂/1♀	9	12	28	31
Decyl acetate	200	—	—	—	—	—	—	—	—	tr	—	—
Dodecyl acetate	228	min	tr	tr	tr	—	min	tr	min	min	—	min
Tetradecyl acetate	256	min	min	min	min	min	—	tr	—	—	min	—
Pentadecyl acetate	270	—	—	—	tr	tr	—	—	—	—	—	—
Hexadecyl acetate	284	maj	maj	maj	maj	maj	maj	maj	—	—	maj	—
Hexadecenyl acetate	282	min	maj	—	maj	maj	—	maj	—	—	—	—
Hexadecadienyl acetate	280	—	—	—	—	maj	maj	—	—	—	—	—
Heptadecenyl acetate	296	tr	tr	—	tr	tr	tr	tr	—	—	—	—
Octadecyl acetate	312	—	—	—	—	—	—	—	—	—	tr	—
Octadecenyl acetate	310	—	min	—	—	min	—	min	—	—	—	—
Octadecadienyl acetate	308	min	min	—	min	min	min	min	—	—	—	—
Dodecyl butanoate	256	—	—	—	—	—	—	—	—	—	—	tr
Decyl 3-methylbutanoate	242	—	—	—	—	—	—	—	tr	—	—	tr
Dodecyl 3-methylbutanoate	270	—	—	—	—	—	—	—	maj	maj	—	maj
Tetradecyl 3-methylbutanoate	298	—	—	—	—	—	—	—	—	min	—	min
Dodecyl hexanoate	284	—	—	—	—	—	—	—	—	—	—	tr
Decyl tetradecanoate	368	—	—	—	—	—	—	—	min	tr	tr	tr
Dodecyl dodecanoate	368	—	—	—	—	—	—	—	min	tr	tr	tr
Dodecyl tetradecanoate	396	—	—	—	—	—	—	—	min	tr	tr	tr
Dodecyl hexadecanoate	424	—	—	—	—	—	—	—	min	tr	—	tr
Dodecyl octadecanoate	452	—	—	—	—	—	—	—	min	tr	—	tr
Tetradecyl dodecanoate	396	—	—	—	—	—	—	—	min	tr	tr	tr
Tetradecyl tetradecanoate	424	—	—	—	—	—	—	—	min	tr	—	tr
Tetradecyl hexadecanoate	452	—	—	—	—	—	—	—	—	tr	—	—
Hexadecyl tetradecanoate	452	—	—	—	—	—	—	—	—	tr	—	—
Hexadecanoic acid	256	—	—	maj	—	—	—	—	tr	—	—	tr
Octadecanoic acid	284	—	—	tr	—	—	—	—	tr	—	—	min
Octadecenoic acid	282	—	—	tr	—	—	—	—	tr	—	—	min
α-Tocopherol	430	tr	tr	—	—	—	tr	—	—	—	—	—
Cholesterol	386	tr	—	tr	tr	tr	tr	tr	tr	—	—	—

Two of the three extracts of the pooled secretions from adult females contained trace amounts of pentadecyl acetate, which was not indicated in samples containing secretions from only adult males.

Identification of the acetates was facilitated by the appearance of CH_3CO^+ and $CH_3CO_2H_2^+$ peaks in their mass spectra at m/z 43 and 61, respectively. The chain length of the alcohol portion was indicated by a peak at $[R-H]^+$ corresponding to: m/z 140 = C_{10}, 168 = C_{12}, 196 = C_{14}, 224 = C_{16}, 252 = C_{18}.

The samples from immature alligators showed none of the unsaturated C_{16} acetates but contained traces of tetradecyl acetate, dodecyl acetate and decyl acetate; the latter was not indicated in the adults' secretions. Immature individuals also included C_{12} and C_{14} 3-methylbutanoates (Figs. 2 and 3) and traces of the C_{10} 3-methylbutanoate; the C_{12} compound was a major component in three of the four pooled samples.

Hexadecyl acetate was indicated as the major component in one sample of immature alligators (28 individuals). This as well as the other three samples from immature alligators contained small amounts of higher molecular weight esters corresponding to mixtures of dodecyl dodecanoate and decyl tetradecanoate (m.w. 368), dodecyl tetradecanoate and tetradecyl dodecanoate (m.w. 396), dodecyl hexadecanoate and tetradecyl tetradecanoate (m.w. 424), and dodecyl octadecanoate, tetradecyl hexadecanoate and hexadecyl tetradecanoate (m.w. 452). These compounds were not detected in the adults.

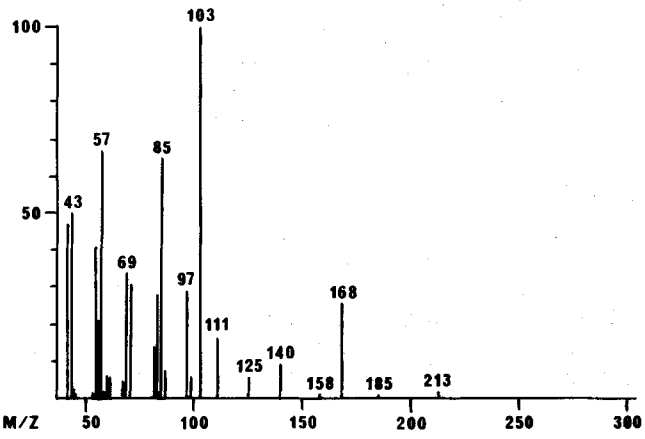

FIG. 2. Mass spectrum of dodecyl 3-methylbutanoate. m/z 85 $[(CH_3)_2CHCH_2CO^+]$, 103 $[(CH_3)_2CHCH_2CO_2H^{2+}]$, 168 $(C_{12}H_{24}^+)$.

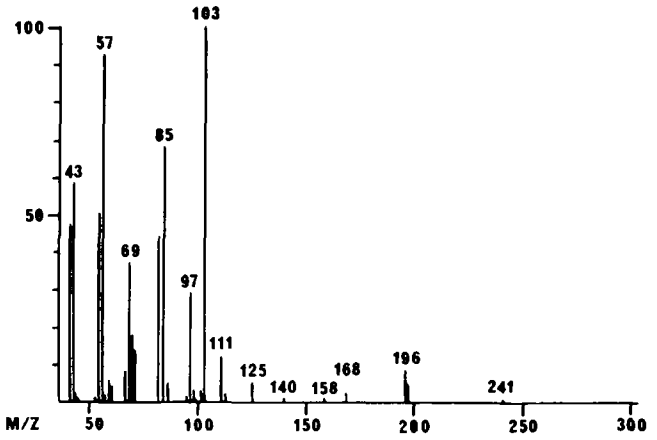

FIG. 3. Mass spectrum of tetradecyl 3-methylbutanoate. m/z 85 [$(CH_3)_2CHCH_2CO^+$], 103 [$(CH_3)_2CHCH_2CO_2H_2^+$], 196 ($C_{14}H_{28}^+$).

Fester and Bertuzzi (2) found that saturated and unsaturated fatty acids were 45% and 35%, respectively, of the water-insoluble saponification products of *Caiman* spp. paracloacal gland secretions. Palmitic (C_{16}), myristic (C_{14}), and isovaleric (C_5) acids were among those detected. Free and esterified constituents, however, were not distinguished. Hexadecanoic acid was detected as a major free fatty acid in a sample of adult male Louisiana *A. mississippiensis* and as a trace component in two pooled samples of immature individuals from Texas. Octadecanoic and octadecenoic acid are trace or minor components in two samples of immature alligators and in one sample of adult males from Louisiana.

Sterols are indicated in a number of analyses of reptile shed skin extracts and integumental gland secretions (5–9). The specific compounds present have not been determined in most of these studies, for which thin layer chromatography or nuclear magnetic resonance of gross secretions are the analytical methods used. Cholesterol occurs in most samples of the paracloacal gland secretions we examined by GC–MS. Steryl esters were indicated by Fester et al. in *Caiman* spp. paracloacal gland secretions (3), but it is as yet unclear whether similar compounds occur in the secretions of other crocodilians.

The fatty alcohols reported in caiman paracloacal gland secretions by Fester and colleagues (2,3) include cetyl alcohol and "yacarol." The latter compound was initially thought to be 2,6-dimethyl-2-hepten-7-ol, but, on the basis of a variety of physical tests, was identified as *d*-citronellol. We did not detect free alcohols in the paracloacal gland secretions of *A. mississippiensis*. The possibility that these compounds occur in alligators or other crocodilians needs to be explored further.

Two dominant peaks in the mass spectrum of one elutant (m/z 430 and 165) indicate α-tocopherol (vitamin E) as a trace component of the secretions of several adult males. The chromatographic retention time and mass spectrum of an authentic sample of α-tocopherol (Aldrich, Milwaukee, WI) confirmed the identity of this vitamin. No other tocols or tocotrienols were detected.

α-Tocopherol, which is produced by higher plants and algae (10), has been detected in the plasma of farm-reared *A. mississippiensis* (11). This compound could be transported to the paracloacal glands via a circulatory or (perhaps from interstitial fluids) a lymphatic route, because elements of both systems have been observed in crocodilian paracloacal glands (1,12–15). The significance of α-tocopherol in alligator paracloacal gland secretions is open to speculation. This substance is known to inhibit lipid oxidation (16,17) and therefore may prevent the degradation of other paracloacal gland compounds.

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Louise Hayes, Kirby Brown and Perry Smith permitted access to alligators in Texas, and Ted Joanen and Larry McNease allowed us to obtain samples from Louisiana alligators. This study was conducted while P.J. Weldon was supported by grants from Texas A&M University and the Whitehall Foundation.

REFERENCES

1. Weldon, P.J., and Sampson, H.W. (1987) *J. Zool. London 212,* 109–115.
2. Fester, G., and Bertuzzi, F. (1934) *Ber. Dtsch. Chem. Ges. 67B,* 365–370.
3. Fester, G., Bertuzzi, F.A., and Pucci, D. (1937) *Ber. Dtsch. Chem. Ges. 70B,* 37–41.
4. Fester, G.A. (1954) *Rev. fac. ing. quim. 23,* 73–74.
5. Burken, R.R., Wertz, P.W., and Downing, D.T. (1985) *Comp. Biochem. Physiol. 81B,* 315–318.
6. Rose, F.L. (1970) *Comp. Biochem. Physiol. 32,* 577–580.
7. Oldak, P.D. (1976) *Copeia 1976,* 320–326.
8. Weldon, P.J., and Bagnall, D. (1987) *Comp. Biochem. Physiol. 87B,* 345–349.
9. Weldon, P.J., and Sampson, H.W. (1988) *Copeia 1988,* 80–86.
10. Draper, H.H. (1980) in *Vitamin E: A Comprehensive Treatise* (Machlin, L.J., ed.) pp. 193–267, Marcel Dekker, New York.
11. Lance, V., Joanen, T., and McNease, L. (1983) *Can. J. Zool. 61,* 1744–1751.
12. Reese, A.M. (1921) *J. Morphol. 35,* 581–599.
13. Pettit, A., and Geay, F. (1904) *C. R. Soc. Biol. 56,* 1087–1089.
14. Voeltzkow, A. (1899) *Abh. Senckenb. Naturforsch. Ges. 26,* 1–149.
15. Wright, D.E., and Moffat, L.A. (1985) in *Biology of Australasian Frogs and Reptiles* (Grigg, G., Shine, R., and Ehmann, H., eds.) pp. 411–422, Surrey Beatty and Sons, Chipping, NSW.
16. McCay, P.B., and King, M.M. (1980) in *Vitamin E: A Comprehensive Treatise* (Machlin, L.J., ed.) pp. 289–317, Marcel Dekker, New York.
17. Burton, G.W., Cheeseman, K.H., Doba, T., Ingold, K.U., and Slater, T.F. (1983) in *Biology of Vitamin E* (Porter, R., and Whelan, J., eds.) pp. 4–14, Pitman, London.

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Distal Small Bowel Resection Does Not Modify the Intestinal 3-Hydroxy-3-methylglutaryl CoA Reductase Activity

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The activity of 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA red.) has been evaluated in rat jejunal, caecal and colonic mucosa adapting to 50% or 75% distal small bowel resection (SBR). Six wk after surgery, the total activity and the activity state (active form) of the enzyme were determined. The results show that HMG-CoA red. activity was stimulated per organ but not per unit mass in the mucosa adapting to SBR. As a side result, we noted regional differences in the active/total reductase ratio (RA/RT) for HMG-CoA red. that was lower in small intestine compared with that of the large intestine.
Lipids 23, 730-732 (1988).

The interruption of the entero-hepatic circulation produces a decrease in the input of bile acids into the liver, leading to an increase in hepatic synthesis of bile acids from cholesterol and hepatic synthesis of cholesterol (1).

Intestinal mucosa commonly is considered to play a secondary role as a source of endogenously synthesized cholesterol. Thus, in the rat, the intestinal mucosa accounts for about 24% of whole-body sterol synthesis, compared with 50% for the liver (2). It has been suggested that under circumstances in which cholesterol needs increase (as in intestinal resection), many extrahepatic tissues may synthesize in situ much of the cholesterol that they require for cholesterol turnover (3). In the liver and intestine, the rate-limiting step for cholesterol biosynthesis is catalyzed by the enzyme 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA red., EC 1.1.1.34) (4,5). In the liver and intestine, the activity of this enzyme could be altered by changes in rates of enzyme synthesis or degradation, or by changes in the activity state of preformed enzyme, as might occur through a phosphorylation-dephosphorylation reaction (6-8). It has been reported that more rapid changes in cholesterol synthesis were linked to the state of enzyme phosphorylation (9).

We have reported that distal small bowel resection (SBR) increases the activity of this enzyme in rat liver (10). This study was designed to evaluate the intestinal cholesterol synthesis determining the total activity of HMG-CoA red., and its active form, in the mucosa of remnant jejunum, caecum and colon in rats after either 50% or 75% distal SBR.

MATERIAL AND METHODS

Animals. Male Wistar rats weighing about 300 g that were maintained on a standard pellet diet with free access to tap water were used. The rats were randomly assigned to one of three groups: sham-operated, 50% and 75% distal SBR. The rats were anaesthetized with intraperitoneal sodium pentobarbitone (4.5 mg/100 g body wt.) after a 24-hr fast period. Laparotomy was performed, and rats assigned for SBR underwent either 50% or 75% SBR, as described by Murillo et al. (11). In another group, sham operation was performed, and the intestine was cut and re-anastomosed without resection. In each instance, continuity of the gut was restored by end-to-end anastomosis. After the surgical operation, the rats were housed in a temperature-controlled laboratory with a strict 4 a.m.-4 p.m./4 p.m.-4 a.m. dark/light cycle. Six wk after SBR, the rats were fasted for 20-24 hr and, between 9 and 10 a.m., to concide with peak nocturnal activity of intestinal HMG-CoA red., were killed by a blow at the base of the skull (4). Both control and experimental groups were treated in the same manner to prevent effects that could mask differences between groups.

Sample preparation procedures. After the animals were killed, the abdomen was opened, and the jejunum, caecum and colon were excised, rinsed free of intestinal contents with ice-cold saline solution and their lengths and weights recorded. The mucosa from each segment was scraped off with a glass slide, weighed and immediately frozen in liquid N_2 and stored at −70 C until the assay of the enzyme. The storage time did not result in significant loss of enzyme activity (unpublished data).

Assay of intestinal enzyme activity. All subsequent operations were carried out at 4 C. The mucosa was homogenized in a Potter-Elvehjem homogenizer with ice-cold homogenization medium containing 0.25 M NaCl, 50 mM imidazole (pH 7.4), 20 mM ethylenediamine tetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) and either 50 mM NaCl (for total reductase activity) or 50 mM NaF (for the active reductase form). In the presence of NaF (an inhibitor of the dephosphorylation), the measured activity of the enzyme reflects the activity that was present initially in the tissue (active form).

Reductase activity was measured essentially as described by Shapiro et al. (12) with light modifications. This method measures the formation of radioactive mevalonate from labeled HMG-CoA. One hundred µl preincubation medium containing 0.2-0.4 mg mucosal protein, in addition to the components present in the homogenization medium, was incubated at 37 C for 15 min. Then, 50 µl of solution containing 0.25 M NaCl, 50 mM imidazole (pH 7.4), 20 mM ethylenediamine tetraacetic acid (EDTA), 5 mM DTT, 110 mM glucose-6-phosphate, 13 mM NADP, 1 unit of glucose-6-phosphate dehydrogenase and 1 mM DL-3-^{14}C-HMG-CoA (specific activity 4000-8000 dpm/nmol), was added to the preincubation mixture and incubated for 30 min at 37 C. The reaction was stopped by adding 25 µl of 5 N HCl. The ^{14}C-mevalonate formed was converted into the labeled lactone, isolated by thin layer chromatography and counted by liquid scintillation using an internal standard of ^{3}H-mevalonate to correct for incomplete recovery.

Protein assay. Mucosal protein was determined by the method of Lowry et al. (13), with bovine serum albumin as the standard.

*To whom correspondence should be addressed.

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; RA/RT, active/total reductase ratio; SBR, small bowel resection.

Materials. All radiochemicals for the assay of HMG-CoA red. activity were obtained from Amersham (UK). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis. The two-tailed Student's t-test was used to evaluate the statistical significance of the differences in the values obtained.

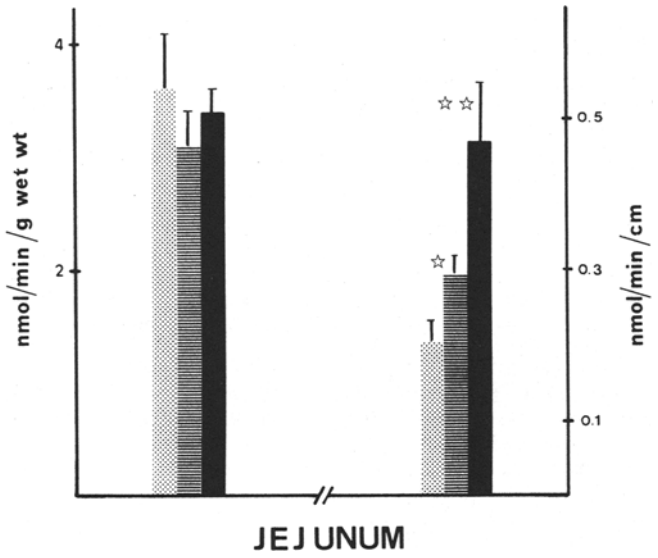

FIG. 1. HMG-CoA reductase activity in jejunal mucosa after 50% (stripes) and 75% (solid) distal SBR, compared with sham rats (dots). Results are given as means ± S.E. of 10 animals. ☆, $p < 0.025$; ☆☆, $p < 0.01$.

RESULTS AND DISCUSSION

We have shown (10) that in rats, both total hepatic activity of HMG-CoA red. and the ratio of active to total reductase (RA/RT) were significantly increased after 50% and 75% SBR. The present data shows that SBR increased both jejunal (Fig. 1) and colonic (Fig. 2) total activity of the enzyme, expressed as nmol/min/cm. However, when the data was referred to unit tissue mass (nmol/min/g wet tissue), the differences between groups were abolished (Fig. 1 and Fig. 2). Therefore, the increased activity was due entirely to intestinal tissue growth induced by the surgical operation (14). In the caecum, HMG-CoA red. total activity expressed per organ was significantly increased after the surgical operation (Fig. 3). This increase again was due to the larger mucosal mass (14), because reference to unit mass (nmol/min/g wet tissue) abolished the differences between groups (Fig. 3). As indicated in Methods, these results were obtained in the fasting state. The response in animals on an ad libitum intake is not known.

Although no change in the total activity of the enzyme was observed in any of the intestinal segments tested, a change in the activity state could have occurred. Therefore, we considered it of interest to investigate whether the state of enzyme phosphorylation was modified by SBR. In the jejunum of sham animals, we found a RA/RT ratio of 0.16, which was not significantly modified by SBR (Table 1), but was significantly lower than that observed in isolated rat enterocytes (8) or in intestinal microsomes (15). These differences could be due to the different preparations used. In caecum and colon of sham rats, this ratio was higher than in jejunum, i.e., 0.56 and 0.57, respectively. Again, this ratio was not changed by SBR (Table 1).

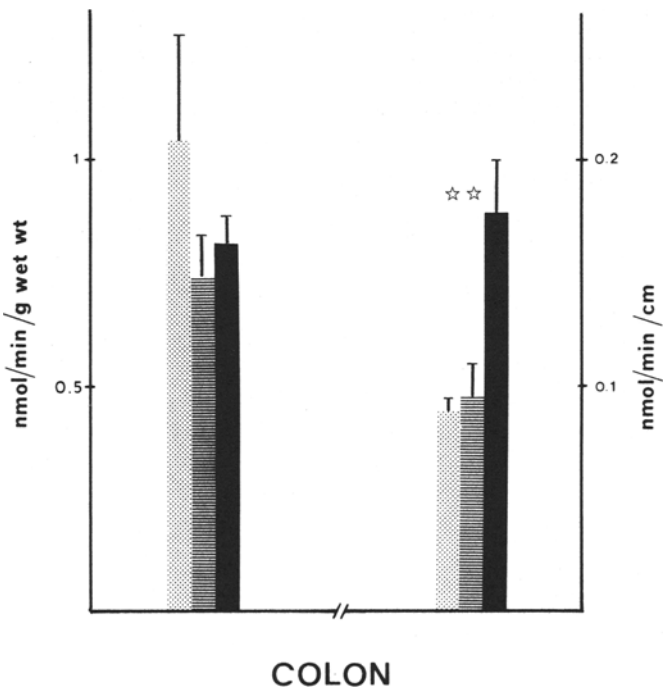

FIG. 2. HMG-CoA reductase activity in colonic mucosa. Results are given as means ± S.E. of 10 animals. Other details as in Figure 1. ☆☆, $p < 0.01$.

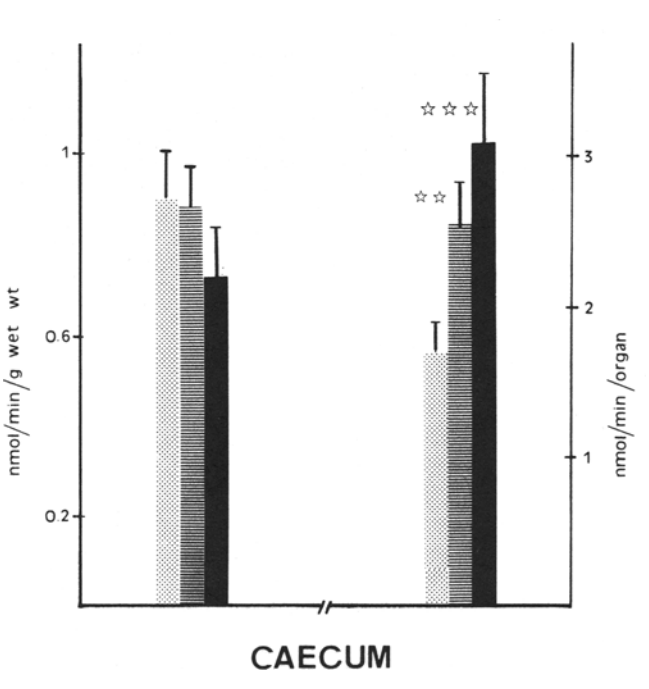

FIG. 3. HMG-CoA reductase activity in caecal mucosa. Results are given as means ± S.E. of 10 animals. Other details as in Figure 1. ☆☆☆, $p < 0.005$.

TABLE 1

Active/Total Reductase (RA/RT) in Jejunal, Caecal and Colonic Mucosa[a]

	Jejunum	Caecum	Colon
Sham	0.16 ± 0.02	0.56 ± 0.03	0.57 ± 0.01
50%	0.15 ± 0.01	0.53 ± 0.02	0.55 ± 0.02
75%	0.14 ± 0.02	0.50 ± 0.03	0.58 ± 0.02

[a]Results are given as means ± S.E. of 10 animals.

Taken together, these results show that SBR altered neither the total activity nor the activity state of the enzyme. An increase in the activity of the intestinal reductase has been observed when the entero-hepatic circulation has been interrupted by either administration of the bile acid-binding resin, as cholestyramine (16) or by external diversion of the bile (17). This discrepancy could be due to differences in the experimental procedures. Thus, in the latter studies bile acid recirculation is interrupted before uptake by the mucosa, while in the current experiments the area of intestinal uptake is reduced as are the sites for bile acid and cholesterol absorption.

We have reported that SBR increases hepatic HMG-CoA red. activity (10). This observation, together with the present findings, might suggest that hepatic activity may be sufficient to supply the majority of cellular needs for cholesterol in resected animals. Other possible causes could contribute to explain the current observations after intestinal resection, such as increased intestinal absorption of cholesterol, increased cellular lipoprotein uptake in intestinal epithelium, etc. However, the possibility that the utilization of newly synthesized cholesterol for chylomicron formation may be the major usage of the intestinal cholesterol cannot be ruled out. These possible causes are under investigation in our laboratory.

In conclusion, this study demonstrates that intestinal HMG-CoA red. does not change in rat jejunal, caecal and colonic mucosa adapting to SBR. As a side result, we noted regional differences in the RA/RT ratio for HMG-CoA red. that was lower in mucosa scraped off the small intestine compared with that of the large intestine.

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REFERENCES

1. Kuipers, F., Havinga, R., Bosschieter, G.P., Toorop, G.P., Hindriks, F.R., and Vonk, R.J. (1985) *Gastroenterology 88,* 403-411.
2. Tarley, S.D., Anderson, J.M., and Dietschy, J.M. (1980) *J. Lipid Res. 22,* 551-569.
3. Spady, D.K., and Dietschy, J.M. (1983) *J. Lipid Res. 24,* 303-315.
4. Rodwell, V.N., and Nordstrom, J.L. (1976) *Adv. Lipid Res. 14,* 1-74.
5. Balasubramanian, S., Goldstein, J.L., and Brown, M.S. (1976) *Proc. Natl. Acad. Sci. USA 73,* 2564-2568.
6. Nordstrom, J.L., Rodwell, V.W., and Mitschelen, J.J. (1977) *J. Biol. Chem. 252,* 8924-8934.
7. Stange, E.F., Alavi, M., Schneider, A., Preclik, G., and Ditschnueit, H. (1980) *Biochim. Biophys. Acta 620,* 520-527.
8. Panini, S.R., and Rudney, H. (1980) *J. Biol. Chem. 255,* 11633-11636.
9. Scallen, T.J., and Sanghvi, A. (1983) *Proc. Natl. Acad. Sci. USA 80,* 2477-2480.
10. Molina, M.T., Murillo, M.L., and Vázquez, C.M. (1986) *IRCS Med. Sci. 14,* 901-902.
11. Murillo, M.L., Campos, M.S., Murillo, A., and Varela, G. (1978) *Rev. Esp. Fisiol. 41,* 359-362.
12. Shapiro, D.J., Nordstrom, J.L., Mitschelen, J.J., Rodwell, V.W., and Schimke, R.T. (1974) *Biochim. Biophys. Acta 370,* 369-377.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
14. Vázquez, C.M., Molina, M.T., and Ilundain, A. (1987) *Acta Microscop. 10(1),* 57-65.
15. Field, F.J., Erickson, S.K., Shrewsbury, M.A., and Cooper, A.D. (1982) *J. Lipid Res. 23,* 105-113.
16. Panini, S.R., Lehrer, G., Rogers, D.H., and Rudney, H. (1979) *J. Lipid Res. 20,* 879-889.
17. Andersen, J.M., and Dietschy, J.M. (1977) *J. Biol. Chem. 252,* 3646-3651.

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Lipids

4th Separation Science and Biotechnology Symposium (August 31–September 3, 1988, Palazzo Feltrinelli, Gargnano del Garda, Italy). Requests for information should be sent to Chairman, Prof. Pier Giorgio Righetti, c/o Euro Business Center, P.O. Box 10552, 1001 EN Amsterdam, The Netherlands.

10th Anniversary Meeting of the European Association for Cancer Research (University College, Galway, Ireland, September 11–13, 1989). For further information, contact Dr. S. M. Lavelle, Experimental Medicine, University College, Galway, Ireland; or in North America, contact Dr. J. H. Weisburger, American Health Foundation, Valhalla, New York, NY 10595-1599. The program involves plenary lectures, workshops, symposia and poster sessions.

The 29th International Conference on the Biochemistry of Lipids (September 19–22, 1988, Tokyo, Japan). Sponsored by The Japanese Biochemical Society and the Foundation for Advancement of International Science. For more information, contact Prof. Y. Seyama, Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

Genetic Basis for Obesity and Diabetes (October 30–November 1, 1988, Stouffer Pineisle Resort, Lake Lanier Islands, Georgia). Sponsored by the University of Georgia, Center for Continuing Education and the College of Home Economics. For more information, contact Dr. Carolyn D. Berdanier, Conference Chair, University of Georgia, Department of Foods and Nutrition, Dawson Hall, Athens, GA 30602.

Vitamin E: Biochemistry and Health Implications (October 31–November 3, The Sheraton Centre, New York, NY). Sponsored by The New York Academy of Sciences. The deadline for submission of abstracts for posters is June 30, 1988. Send the 200-word summary to Dr. Lawrence J. Machlin, Clinical Nutrition, Hoffman-La Roche Inc., Nutley, NJ 07110. For more information, contact the Conference Department, The New York Academy of Sciences, 2 East 63rd St., New York, NY 10021, (212) 838-0230.

International Symposium on Clinical, Biochemical and Molecular Aspects of Fatty Acid Oxidation (November 6–9, Penn Tower Hotel, Philadelphia, PA). For more information, contact Paul M. Coates, Ph.D., Division of Genetics, The Children's Hospital of Philadelphia, 34th St. and Civic Center Blvd., Philadelphia, PA 19104.

Cell Membrane Localization of Long Chain C_{24}-C_{30} Fatty Acids in Two Marine Demosponges[1]

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Subcellular fractionation by differential centrifugation was performed on two previously unstudied marine sponges (*Reniera* sp. and *Pseudaxinyssa* sp.) that represent both major subclasses of the Demospongiae. Long chain fatty acids (LCFA) with 24-30 carbon units were found as major constituents of cell membrane isolates of both sponges. Most LCFA were polyunsaturated and were constituents of the phospholipids, which are typical membrane lipids, and in particular the amino-phospholipids. The LCFA composition of phospholipids from whole sponge tissue was shown to provide a reliable indication of the LCFA composition of cell membrane phospholipids in the sponges studied. An unusual triply branched C_{16} isoprenoid fatty acid, 4,8,12-trimethyltridecanoic acid, also was identified as a cell membrane acid in the sponge *Pseudaxinyssa* sp.
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Long chain fatty acids (LCFA) with molecular chains of 24-30 carbon units have been identified as major components of cellular membranes in one marine sponge (1). In the aforementioned study, 51% of the fatty acids in a mixed membrane isolate from the intertidal species *Halichondria moorei* were LCFA with chain lengths in excess of the 14-22 carbon units which usually occur in the membranes of eucaryotic cells (2). Sponges are renowned for their content of LCFA, which can comprise up to 85% of the total fatty acids present in their tissue (3-10); in the few sponge species that have been studied, most LCFA were found localized in phospholipids (1,5,11-15).

No studies, however, yet have unambiguously established the presence of LCFA in sponge cells; all studies have employed sponge tissue or membrane preparations potentially contaminated by microorganism symbionts (e.g., bacteria and unicellular algae) that typically inhabit the matrix region of sponge tissue (16-18). While bacteria typically contain fatty acids with chain lengths up to 22 carbons (2,19-23), there are rare instances in which bacterial species contain high molecular weight fatty acids (e.g., mycobacteria produce β-hydroxy fatty acids with between 60 and 83 carbons [24]). Moreover, there is little information on the identities of other microorganism sponge symbionts or on their fatty acid contents.

Sponges also are unique in the diversity and often novelty of their sterols (25-31). While there are sponge species that solely contain conventional cholesterol-type sterols, which are typical of eucaryotes (32), many sponges substitute unusual sterols (e.g., unusual patterns of alkylation and unsaturation in the C_{17} side chain). Because sterols are major components of cellular membranes in almost all eucaryotic organisms, it is expected (28) that such unconventional sterols assume important structural positions in sponge membranes, but it is unknown whether these unusual sterols co-occur with LCFA in sponge membranes.

Therefore, the purpose of this study was to determine unambiguously whether LCFA are components of sponge membranes, while the companion study (33) seeks to establish whether unconventional sterols also are sponge membrane components. Here the subcellular fractionation study of Lawson et al. (1) is extended to include two additional species that together represent both major subclasses of the Demospongiae, with one species that lacks discernible matrix symbionts and contains conventional sterols, and another species that is noted for its high matrix symbiont content and unconventional sterols.

The California ceractinomorph sponge *Reniera* sp. is seasonally abundant (April-July) in the San Francisco Bay. Specimens of this sponge that are devoid of microorganism symbionts can be obtained during early rapid stages of growth April-May (Fig. 1a). *Reniera* sp. contains LCFA that often have Δ5,9 unsaturation (described herein) and conventional sterols (33).

The Great Barrier Reef tetractinomorph sponge, *Pseudaxinyssa* sp., is a long-lived animal that contains considerable bacterial populations, especially phototropic cyanobacteria (= blue-green algae), in the illuminated surface (<1 mm) tissues (18, unpublished data, this laboratory). *Pseudaxinyssa* sp. contains LCFA that often have Δ5,9 unsaturation patterns (unpublished data, this laboratory) and unconventional sterols with an isopropyl branch at C-24 of the side chain (27,33-39).

EXPERIMENTAL

Sponge samples. Reniera sp. (Order Haplosclerida, California Academy of Sciences Museum specimen #063176) was collected by hand from San Francisco Bay, CA (3 m) in April 1985. *Pseudaxinyssa* sp. (Order Axinellida, Australian Museum specimen #Z4988) was collected by hand from the John Brewer Reef, Australian Great Barrier Reef (18 m) in June 1985 (for biological and chemical studies) and July 1986 (for further electron microscopy [EM] studies).

Chemicals and materials. Electron microscopy reagents were EM grade from either Electron Microscopy Sciences (Fort Washington, PA), Ted Palla Inc. (Tustin, CA) or Ernest F. Fullam Inc. (Latham, NY). Phospholipid reference compounds included phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidyl-

[1]Part 16 of "Phospholipid Studies of Marine Organisms." For Part 15 in this series see Dasgupta, A., Ayanoglu, E., Tomer, K.B., and Djerassi, C. (1987) *Chem. Phys. Lipids 43*, 101-111.

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Abbreviations: BHT, butylated hydroxytoluene; EDTA, ethylenediamine tetraacetic acid; FAME, fatty acid methyl ester; LCFA, long chain fatty acid; LCPUFA, long chain polyunsaturated fatty acid; LCUFA, long chain unsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; TMTD, 4,8,12-trimethyltridecanoic acid; UFA, unsaturated fatty acid; A, archaeocytes; CC, choanocyte chambers; ECL, equivalent chain length; EM, electron microscopy; F, flagella; FSW, filtered seawater; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; MV, microvilli.

glycerol, diphosphatidylglycerol, phosphatidic acid, phosphatidylinositol, lysophosphatidylcholine, phosphatidylethanolamine plasmalogen, and sphingomyelin, and were from Sigma Chemical Co. (St. Louis, MO). Other reference lipids were cholesterol (Aldrich-Milwaukee, WI), cholesteryl myristate (Sigma Chemical Co.), and tripalmitin (Sigma Chemical Co.). General laboratory reagents were purchased from May & Baker Pty. Ltd, Sigma and Aldrich Chemical Companies. All solvents were analytical grade and were distilled before use.

Thin layer chromatography (TLC) plates (silica coated aluminium, Kieselgel 60 F254, 0.2 mm, Merck Chemical Co., Darmstadt, Germany) were prewashed in diethylether before use. Fatty acid methyl esters (FAME) were purified on Davisil 62 silica gel.

Subcellular fractionation. Subcellular fractionation of sponge tissue was modified after Lawson et al. (1). Filtered seawater (FSW, 10 C, 0.4μ nucleopore filter) was used throughout the procedure. *Reniera* sp. (300 g, 20 specimens) and *Pseudaxinyssa* sp. (200 g portion of 1 individual) were chopped into pieces (1 cm^3) and placed in cold homogenizing medium (50 mM Tris, 250 mM sucrose, 5 mM ethylenediamine tetraacetic acid [EDTA]/FSW, 10 C, pH 7.4, 0.5 ml/g tissue). Sponge tissue was homogenized in a Galaxie Osterizer dual-range 14 kitchen blender at high speed for 40 sec for *Reniera* sp. and in a Sorvall Omni-mixer (model 17220) at 16,000 rpm (high speed) for 80 sec for *Pseudaxinyssa* sp; homogenization conditions were restricted to minimize disruption of intracellular organelles. *Pseudaxinyssa* tissue did not completely homogenize, as did *Reniera* tissue, due to its resilient siliceous and collagenous skeleton. Homogenates were filtered through nylon mesh (200μ). Residual *Pseudaxinyssa* sp. tissue, which remained after filtration, was found to have a lipid content similar to that of whole sponge. Filtrates were diluted in homogenizing medium (1:1, v/v) and were successively centrifuged at 600 × g/15 min/10 C, 15,000 × g/15 min/10 C (Sorvall RC 5 centrifuge, GSA rotor for *Reniera* sp; Beckman J-21B centrifuge, JA14 rotor for *Pseudaxinyssa* sp.) and 100,000 × g/1 hr/4 C (Beckman L8-70M ultracentrifuge, 70.1 Ti rotor for *Reniera* sp; ICE/B-60 ultracentrifuge, A21 rotor for *Pseudaxinyssa* sp.). For each sponge, the three successive tissue pellets that are referred to according to convention as P1, P2 and P3, respectively, and a final supernatant resulted from these respective centrifugations. P1 was washed once in homogenizing medium, and P2 was washed twice. All fractions were held on ice throughout the procedure.

Electron microscopy. Fresh tissue samples from whole sponge and pelleted subcellular fractions were fixed in 2.5% glutaraldehyde/0.4 M sodium cacodylate buffer/10 C, post-fixed in 1% osmium tetroxide in the same buffer, and embedded in epoxy resin (40). Silver sections were stained with 1% uranyl acetate and lead citrate and were examined at 2,250, 7,500, 30,000, and 138,000 × magnification with a Philips 410 electron microscope (80 kv). The resulting micrographs allowed description of cellular and subcellular components in each sample to be made, although those for *Pseudaxinyssa* sp. were of poor contrast and consequently are not reprinted here.

Lipid analysis. Tissue was frozen and stored at −20 C and, in the case of *Pseudaxinyssa* sp., was lyophilized before workup. Total lipids were extracted (3 times) with chloroform/methanol (2:1) containing 0.002% butylated hydroxytoluene (BHT) (41). Supernatants were extracted in chloroform/methanol (4:1, v/v) at 5 C to facilitate partitioning of aqueous and organic phases. Before analysis, lipids were stored in chloroform/methanol (1:1, v/v) containing 0.002% BHT under argon at −20 C.

A portion of the total lipid extract from *Reniera* sp. whole tissue was separated by column chromatography (SIL-R 100-300 mesh silicic acid, Sigma Chemical Co.) into fractions that conventionally contain triacylglycerols and sterol esters (fraction 1), diacylglycerols and free sterols (fraction 2), monoacylglycerols (fraction 3), glycolipids (fraction 4), and phospholipids (fraction 5), after the methods of Privett et al. (42) and Walkup et al. (11). Fractions 1-3 were eluted with chloroform, fraction 4 with acetone, and fraction 5 with methanol. The composition of eluates was monitored by TLC that utilized codeveloped reference compounds and spray reagents (iodine vapor, rhodamine 6G, ceric sulfate, and molybdenum blue [43]) to identify classes of lipids.

Phospholipids were further separated according to head groups by one- and two-dimensional TLC of a small phospholipid sample. For single-dimension chromatography, the solvent system was chloroform/acetone/methanol/ammonium hydroxide (28% aq.)/water (65:35:4:4:0.2, v/v/v/vv/) (11). For two-dimensional chromatography, tetrahydrofuran/acetone/methanol/water (50:20:40:6, v/v/v/v) was employed in development in the first dimension, and chloroform/acetone/methanol/acetic acid/water (50:20:10:15:5, v/v/v/v/v) in the second dimension (44). Phospholipid head groups were identified from TLC R_f-values and by response to specific spray reagents (ninhydrin, Dragendorff, periodate-Schiff, and 2,4-dinitrophenylhydrazine [45]).

The fatty acyl components of total lipid extracts from whole sponges, P3 membrane, and other subcellular isolates of *Reniera* sp. and *Pseudaxinyssa* sp., and of lipid class fractions from whole sponge extracts of *Reniera* sp., were converted to methyl esters (1.25 N hydrochloric acid/methanol [46]). The resulting FAME were purified by silica gel column chromatography with elution by hexane/ether (15:1, v/v). Portions of FAME samples were hydrogenated (methanol, platinum IV oxide catalyst [47]).

Phospholipids from the P3 membrane isolate of *Reniera* sp. were separated according to head groups and isolated by preparative TLC. The major phospholipid head groups were analyzed for fatty acid composition by transesterification on silica with methanolic boron trifluoride (48). The resulting FAME were purified as above.

FAME were analyzed by temperature-programmed gas chromatography (GC) (cooled column injection system, flame ionization detector) on either a Carlo Erba series 4160 Fractovap chromatograph (25 m × 0.32 mm i.d. SE-54 coated fused silica column; program 130-290 C, 5 C/min; detector temp. 290 C, Omniscribe Series D-5000 recorder) or a Hewlett-Packard 5898A series gas chromatograph (25 m × 0.3 mm i.d. SE-54 coated fused silica column; program 170-320 C, 5 C/min; injector temp. 250 C; detector temp. 320 C), with automatic injection system (automatic sampler Model 7672A), integrator (Model 3392A), sampler/event control module (Model 19405A) and computer fatty acid library (software s/n: 2614R100027, vers. 1.2). For Carlo Erba GC, the non-integrated traces were enlarged (1.5 times) and peak areas

were measured by triangulation for calculation of relative proportions of fatty acid components.

Individual FAME were identified by mass spectrometry of their N-acylpyrrolidide derivatives (treatment of FAME with pyrrolidine/acetic acid [10:1, v/v]) (49). Capillary gas chromatography-mass spectrometry (GC-MS) analyses were performed with a Ribermag R10-10 quadrupole mass spectrometer connected to a Carlo Erba series 4160 Fractovap chromatograph (30 m × 0.32 mm i.d. SE-54 fused silica column), and a Hewlett-Packard 5970 series mass selective detector connected to a 5890A gas chromatograph (12 m × 0.2 mm i.d. SE-30 fused silica column).

RESULTS

Subcellular fractionation. Substantial P1 and P2 isolates were produced from both sponges as numerous subcellular organelles remained intact and sedimented at the P1 and P2 centrifugation steps. For both sponges, P1 consisted of partially disrupted cells and large organelles (>2.8 μ diam.) (Fig. 1b), and P2 consisted of smaller organelles (≤2.8 μ diam.) (Fig. 1c). Large numbers of cyanobacteria also were present in the P2 fraction of *Pseudaxinyssa* sp.

P3 membrane isolates for both sponges contained membrane vesicles (mean diam. 0.42 μ), and nonlipidic ribosomes that should not have interfered with lipid analyses (Fig. 1d and 1e). For *Reniera* sp., the P3 membrane isolate would have been essentially free from microorganism membranes because the experimental specimens used were free of matrix microorganism symbionts and contained only a few bacteria in archaeocyte cell digestive vacuoles (Fig. 1a). For *Pseudaxinyssa* sp., the P3 membrane isolate, however, cannot be assumed free of microorganisms because extracellular cyanobacteria characterized by distinctive thylakoids were observed in abundance in the surface tissues (<1 mm) of the sponge. However, such microorganism membrane contamination would be expected to be minor because many intact cyanobacteria were observed in the P1 and P2 isolates, while few thylakoid fragments were observed in any isolate fraction. The sponge membranes present almost certainly included a mixture of plasma and organelle membranes from the many sponge cell types present, although plasma membranes and endoplasmic reticulum from the abundant archaeocyte cells should have dominated due to the great numbers of intact organelles observed in the denser P1 and P2 isolates.

Lipid analysis. Sponge lipid extracts from whole tissue, P1 and P2 isolates, consisted mainly of pigments, sterol esters, triacylglycerols, sterols and phospholipids, while P3 membrane isolates of both sponges were free of pigments and consisted of sterols, phospholipids and lesser amounts of sterol esters. P1 and, especially, P2 fractions from *Pseudaxinyssa* sp. were enriched in chlorophylls that probably originated in the symbiotic cyanobacteria that sedimented in these isolates. A non-esterifiable yellow compound that gave an orange product upon reacting with the ceric sulfate spray reagent (R_f = 0.16 in hexane/ether [1:1, v/v]) was concentrated in the P2 fraction from *Reniera* sp. An unidentified compound (R_f = 0.57 in hexane/ether [1:1, v/v]) was detected in the P3 membrane isolate from *Pseudaxinyssa* sp. Final supernatant isolates from each sponge contained lipidic and water-soluble pigments and sterol esters.

Whole sponges contained LCFA to 28 carbon units in *Reniera* sp. and to 30 carbon units in *Pseudaxinyssa* sp., including many Δ5,9 unsaturated acids (Tables 1 and 2) largely contained in phospholipids (76% and 42%, respectively). The major fatty acids of *Reniera* sp. were $C_{26:2}$ (23%), $C_{23:3}$ (12%), and $C_{28:3}$ (11%), and those of *Pseudaxinyssa* sp. were $C_{16:0}$ (10%), $C_{28:1}$ (9%), and $C_{18:1}$ (7%) (Fig. 2). High levels of typical bacterial acids ($C_{16:0}$ and $C_{18:1}$) in *Pseudaxinyssa* sp. may reflect the large populations of bacterial symbionts present in this sponge, although these acids also commonly occur in eucaryotic cells (2). For *Pseudaxinyssa* sp. but not *Reniera* sp. LCFA content of whole tissues was almost identical for phospholipid and total lipid extracts (Fig. 2).

P3 membrane isolates of both sponges contained a high proportion of LCFA, largely present in phospholipids (Fig. 2). For the *Reniera* sp., P3 membrane-isolate LCFA composed 62% of the total lipid fatty acids and 79% of the phospholipid fatty acids. In contrast, for the *Pseudaxinyssa* sp., P3 membrane isolate LCFA equaled 63% of the total lipid fatty acids but only 37% of the phospholipid fatty acids (Fig. 2), suggesting that LCFA also were abundant in other membrane-associated lipid classes in this sponge. For both sponges, P1 and P2 isolates contained a lower proportion of LCFA. Less than 10% of fatty acids in supernatant isolates from *Reniera* sp. and less than 5% of fatty acids in supernatant isolates from *Pseudaxinyssa* sp. were LCFA.

The presence of LCFA in other lipid classes was demonstrated for whole tissues in *Reniera* sp. in lesser amounts than were detected in phospholipids (Fig. 3). Lipids with chromatographic patterns (42) similar to sterol esters and triacylglycerols (Fraction 1), diacylglycerols (Fraction 2) and monoacylglycerols (Fraction 3) contained 55%, 62% and 7% LCFA, respectively. A predominance of mid-range fatty acids between C_{22} and C_{24} chain lengths (67%) characterized lipids with chromatographic patterns like glycolipids (Fraction 4). Insufficient sponge tissue was available to conduct similar analyses for *Pseudaxinyssa* sp., but it is known from earlier studies on this sponge that LCFA also are concentrated in the phospholipids (unpublished data, this laboratory).

It was notable that for *Reniera* sp. the proportions of LCFA from both phospholipid and total lipid extracts of the P3 membrane isolates were virtually identical (i.e., within 2%) to the respective proportions of LCFA in phospholipid and total lipid extracts from the whole animal (Fig. 2). Such a trend has been noted before in the intertidal sponge *Halichondria moorei* (1) but was not observed in *Pseudaxinyssa* sp. where the P3 membrane isolate contained appreciably more LCFA in the total lipid extract (63%) than did whole sponge tissue (41%), likely due to the substantial numbers of microorganism symbionts present in the whole sponge. Thus, in sponges where symbionts are absent or present in relatively small populations, as in *Halichondria moorei* and *Reniera* sp., the fatty acid composition of whole sponge phospholipids may provide a useful approximation to the LCFA composition of sponge cell membrane phospholipids, while the total lipid composition also may reflect that of the membrane isolate, assuming the sponge does not store significant concentrations of fatty acids in intracellular organelles.

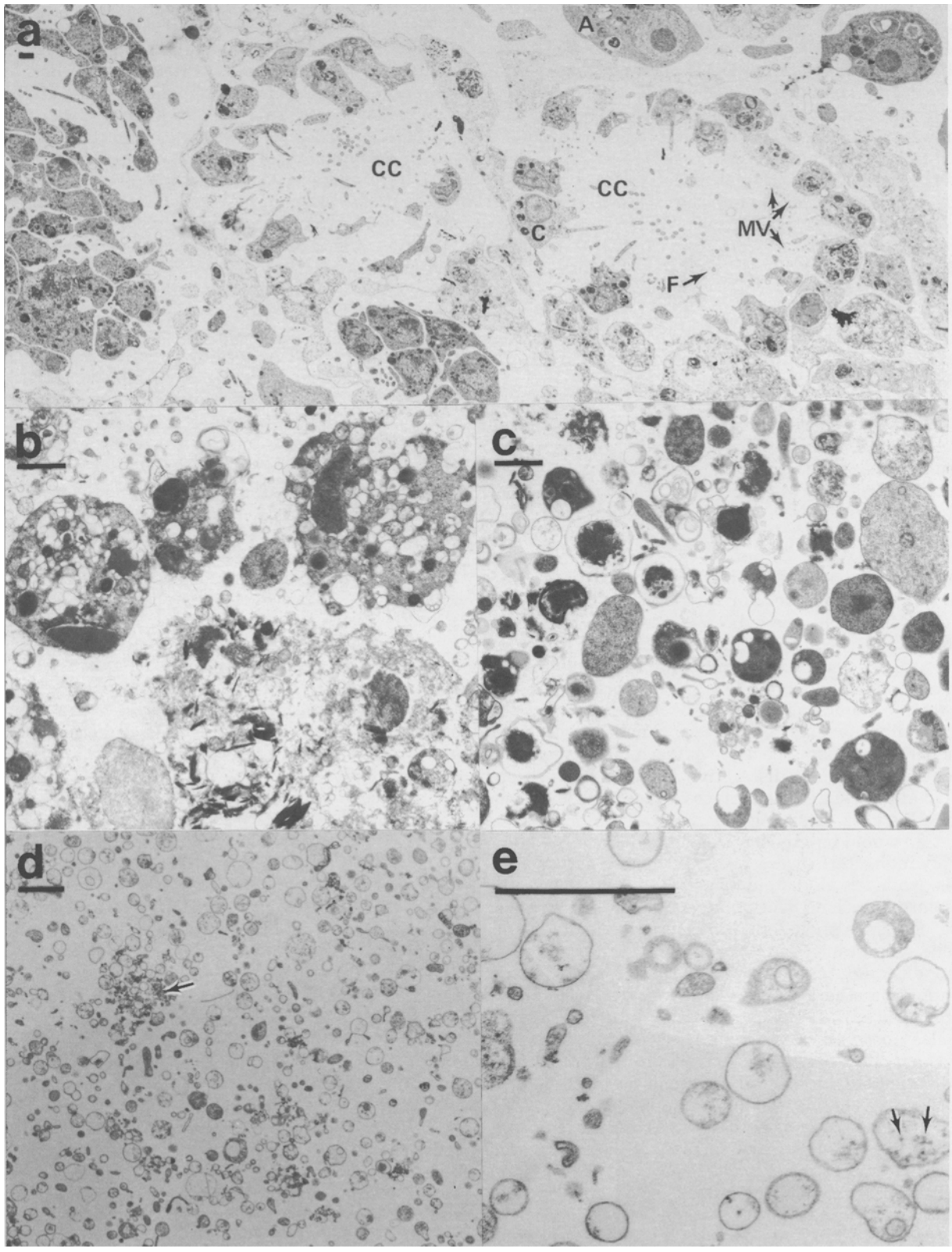

FIG. 1. Electron micrographs of whole sponge and subcellular tissue from *Reniera* sp. (a) Whole sponge tissue contained numerous choanocyte chambers (CC) with component choanocytes (C) of which flagella (F), and microvilli (MV) are obvious. Heavily vacuolated archaeocytes (A) were present in the mesohyl matrix. Mean archaeocyte plasma membrane width 5.3 nm. (b) The P1 subcellular fraction contained partially disrupted cells, and large organelles (>2.8 μ diam.). (c) The P2 fraction contained smaller organelles (≤2.8 μ diam.). (d,e) The P3 fraction contained membrane vesicles (mean vesicle diam. 0.42 μ; mean membrane thickness 5.3 nm) and ribosomal contaminants (arrows). Each bar equals 1 μ.

TABLE 1

Fatty Acids Present in *Reniera* sp. According to Equivalent Chain Length (ECL), as Determined by GC

Peak number	ECL	Identity	Peak number	ECL	Identity
1	13.997	C14:0	30	21.482	Unknown
2	14.398	Unknown	31	21.663	Unknown
3	14.494	C16:0br (TMTD)	32	21.701	Unknown
4	14.621	C15:0br	33	21.793	Unknown
5	14.713	C15:0br	34	21.849	C22:1
6	14.999	C15:0	35	22.001	C22:0
7	15.152	Unknown	36	22.635	Unknown
8	15.203	C14:0-2OH	37	22.854	Unknown
9	15.489	C14:0-2OH/C16:1br	38	22.908	C23:1
10	15.627	C16:0br	39	23.001	Unknown
11	15.817	C16:1 *cis* 9	40	23.425	Unknown
12	16.001	C16:0	41	23.670	C24:1
13	16.337	Unknown	42	23.793	C24:1
14	16.720	C17:0br	43	23.865	C24:1
15	16.998	C17:0	44	24.002	C24:0
16	17.153	Unknown	45	24.299	Unknown
17	17.190	Unknown	46	24.667	Unknown
18	17.234	C16:0-2OH	47	25.001	C25:0
19	17.401	Unknown	48	25.523	C26:3
20	17.652	Unknown	49	25.651	C26:2 *cis* 5,9
21	17.771	C18:1 *cis* 9	50	25.697	C26:0br
22	17.824	C18:1	51	25.752	C26:0br
23	17.999	C18:0	52	25.802	C26:1 *cis* 9
24	18.998	C19:0	53	25.886	Unknown
25	19.401	C20:4 *cis* 5,8,11,14	54	26.002	C26:0
26	19.475	C20:4 *cis* 6,9,12,15	55	26.660	C27:2 *cis* 5,9
27	19.816	C20:1	56	27.479	C28:3
28	19.997	C20:0	57	27.564	C28:2 *cis* 5,9
29	21.352	C22:6	58	27.671	C28:1 *cis* 9

TMTD, 4,8,12-trimethyltridecanoic acid.

TABLE 2

Fatty Acids Present in *Pseudaxinyssa* sp. According to Equivalent Chain Length (ECL), as Determined by GC

Peak number	ECL	Identity	Peak number	23.788	Identity
1	13.393	C14:1br	22	23.788	C24:1
2	14.001	C14:0	23	23.861	C24:1
3	14.488	C16:0br (TMTD)	24	24.661	Unknown
4	15.000	C15:0	25	24.996	C25:0
5	15.818	C16:1 *cis* 9	26	25.651	C26:2 *cis* 5,9
6	15.999	C16:0	27	25.998	C26:0
7	16.421	C17:1br	28	26.395	C26:0
8	16.999	C17:0	29	26.395	Unknown
9	17.190	Unknown	30	26.651	C27:2 *cis* 5,9
10	17.611	C18:2 *cis* 5,9	31	26.997	C27:0
11	17.719	C18:2 *cis* 9,12	32	27.489	C28:3 *cis* 5,9,21
12	17.770	C18:1 *cis* 9	33	27.561	C28:2 *cis* 5,9
13	18.000	C18:0	34	27.639	C28:1
14	19.000	C19:0	35	27.641	Unknown
15	19.608	C20:2 *cis* 11	36	27.899	Unknown
16	19.705	C20:1	37	27.999	C28:0
17	19.773	C20:1 *cis* 11	38	28.620	C29:2
18	19.999	C20:0	39	29.000	C29:0
19	21.399	C22:4	40	29.510	C30:3 *cis* 5,9,23
20	21.557	C22:4	41	30.000	C30:0
21	21.693	Unknown			

TMTD, 4,8,12-trimethyltridecanoic acid.

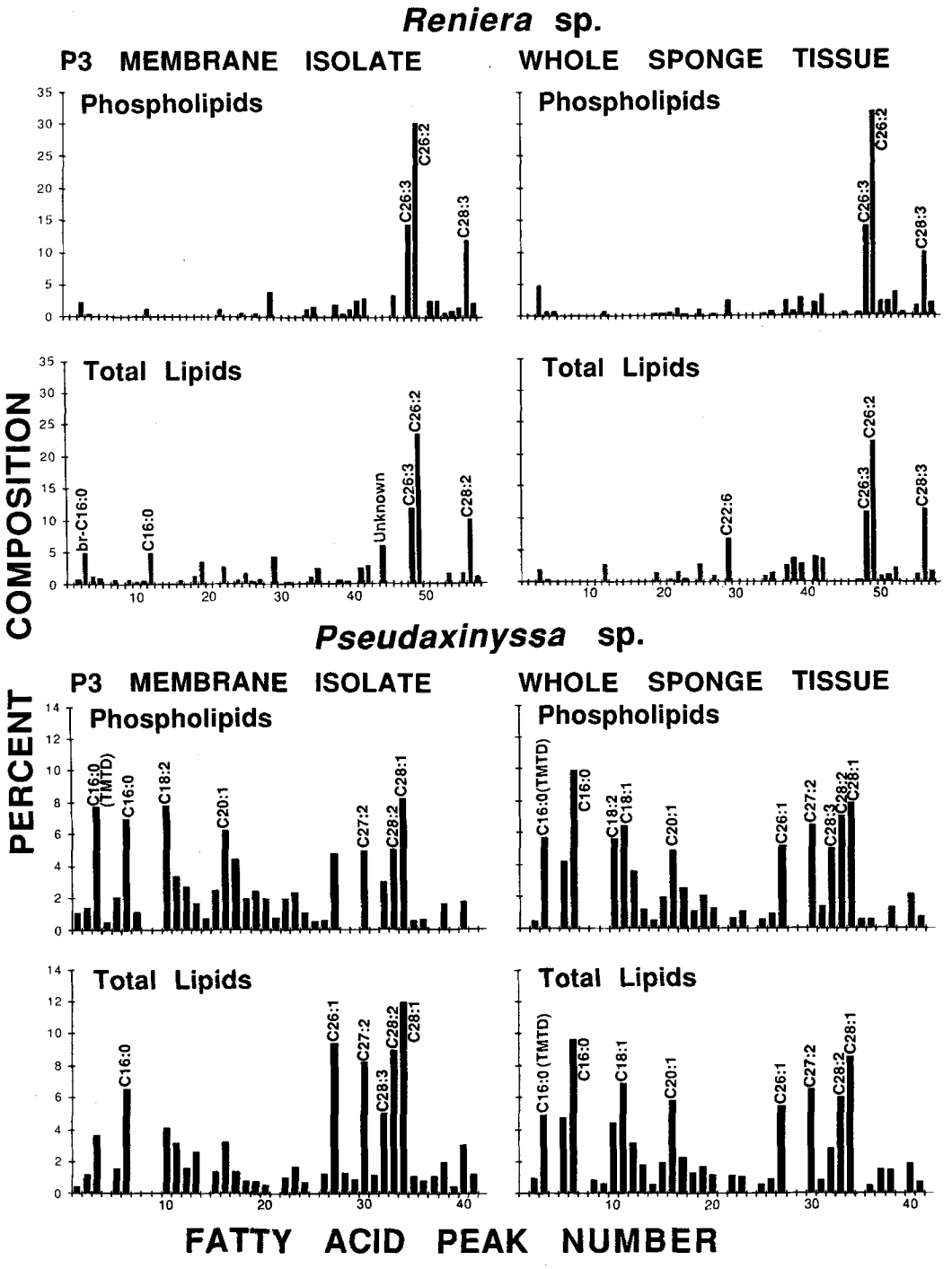

FIG. 2. The proportional distributions of fatty acids in phospholipid and total lipid extracts from P3 membrane isolates and whole sponge tissues of *Reniera* sp. and *Pseudaxinyssa* sp. Fatty acids are described for *Reniera* sp. in Table 1 and for *Pseudaxinyssa* sp. in Table 2. Fatty acids that compose ≥5% of the total fatty acids are labeled.

The LCFA encountered in all tissue samples were predominantly unsaturated, mainly polyunsaturated, while shorter chain fatty acids tended to be saturated (Fig. 4). Such features of unsaturation and polyunsaturation in LCFA are commonly observed in sponges (4,9,10), but it should be noted that the content of LCFA and the degree of unsaturation in individual species of sponge can vary with seasonal temperature (5,50) and with geography (51).

For *Pseudaxinyssa* sp., the unusual isoprenoid fatty acid 4,8,12-trimethyltridecanoic acid (TMTD) was more abundant in the phospholipids of the P3 membrane isolate (7.8%) than in the whole sponge (5.8%) (Fig. 2). This result is in agreement with our unpublished data that documents lesser concentrations of TMTD in the cyanobacteria-rich outer tissues of the sponge.

The principal phospholipids in *Reniera* sp. and *Pseudaxinyssa* sp. whole sponge and P3 membrane isolates were phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) as is typical of those sponges that have been studied for phospholipid content

(5,11,12,52). In all *Reniera* sp. lipid samples, PS appeared as two almost distinct spots on TLC and this may indicate that there are sufficient differences in chain lengths and unsaturation of the fatty acid substituents of PS to result in separation by TLC. In addition to PS, PE and PC, *Pseudaxinyssa* sp. whole sponge tissue and the P1 and P2 isolates contained phosphatidylglycerol, diphosphatidylglycerol and phosphatidylinositol, the former two phospholipids being typical bacterial phospholipids (2).

For the *Reniera* sp., P3 membrane isolate LCFA predominated in amino-phospholipids where PE contained 78% LCFA, and PS contained 58% LCFA (Fig. 5). Almost 40% of the fatty acids in PE consisted of $C_{26:2}$ with $C_{26:3}$ and $C_{28:3}$ accounting for 14 and 13% of the total, respectively. In PS, $C_{26:2}$ was present as 33% of the total and $C_{22:0}$ at 22%. Short chain fatty acids characterized PC with $C_{16:0}$ (37%), $C_{18:0}$ (28%), and $C_{22:6}$ (16%) as the major acids. Insufficient sample was available to perform similar analyses for *Pseudaxinyssa* sp.

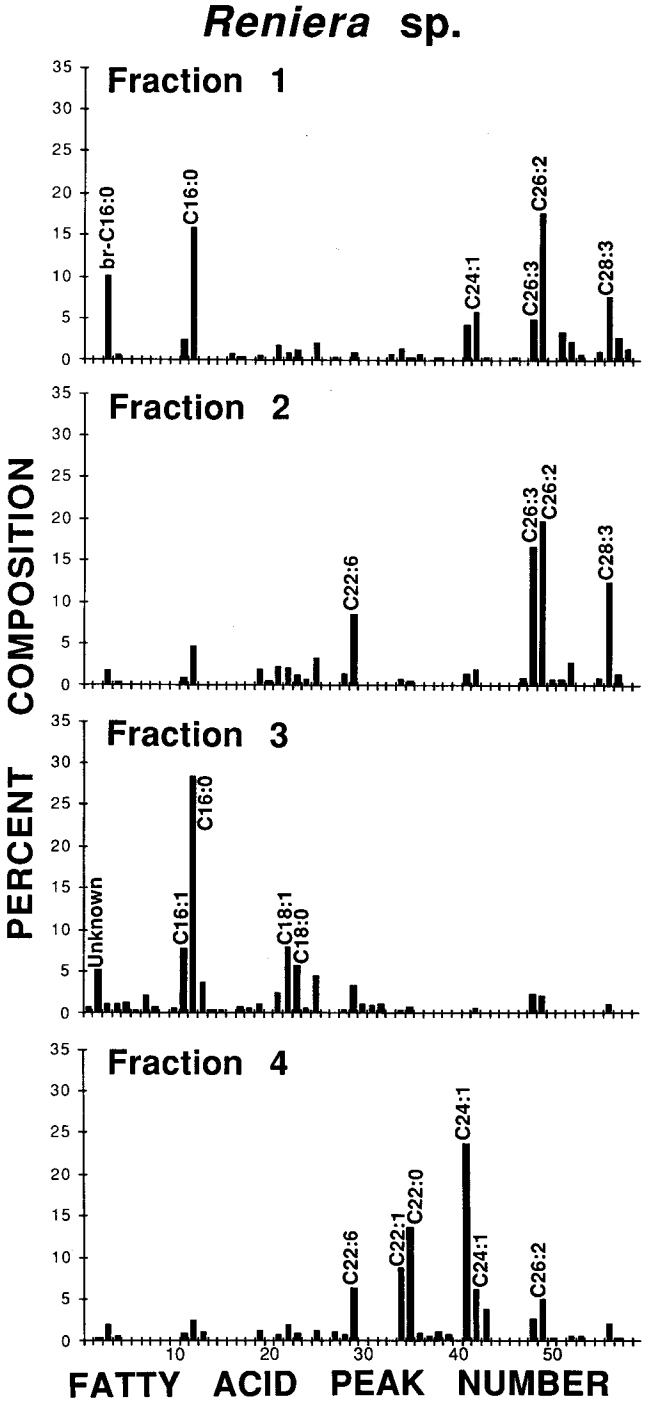

FIG. 3. The proportional distributions of fatty acids in lipid fractions from whole sponge extracts of *Reniera* sp., according to standard column chromatography methods that typically yield fractions of the following compositions: Fraction 1 = triacylglycerols and sterol esters; Fraction 2 = diacylglycerols and free sterols; Fraction 3 = monoacylglycerols; Fraction 4 = glycolipids. Fatty acids are described in Table 1. Fatty acids that compose ≥5% of the total fatty acids are labeled.

DISCUSSION

There now can be no doubt that fatty acids with chain lengths of 24-30 carbons are components of sponge cellular membranes, principally in phospholipids, in addition to appreciable amounts of shorter chain fatty acids. Including this study, three geographically and environmentally distinct sponge species now have been found to contain long chain fatty acids (LCFA) in their cell membranes (1). Moreover, the presence, quantity and nature of mesohyl matrix symbionts do not appear to influence whether a sponge contains LCFA in its cell membranes: *Halichondria moorei* (1) and *Reniera* sp. contained few or no bacterial symbionts, while *Pseudaxinyssa* sp. contained large populations of both phototrophic and nonphototrophic bacterial symbionts; all of these sponges contained LCFA in their cell membranes.

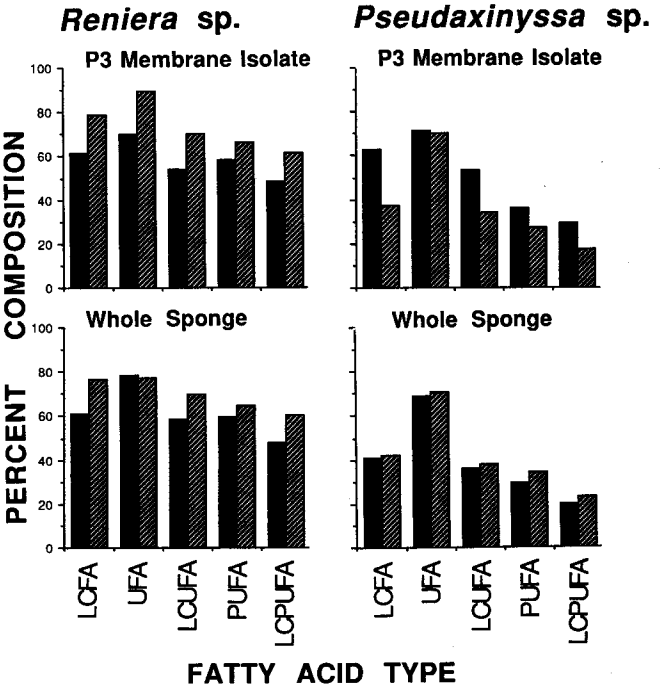

FIG. 4. A comparison of the distribution of long chain and unsaturated fatty acids in total lipids (solid) and phospholipids (hatched) from P3 membrane isolates and whole sponge tissue of *Reniera* sp. and *Pseudaxinyssa* sp. LCFA, long chain fatty acids; UFA, unsaturated fatty acids; LCUFA, long chain unsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCPUFA, long chain polyunsaturated fatty acids.

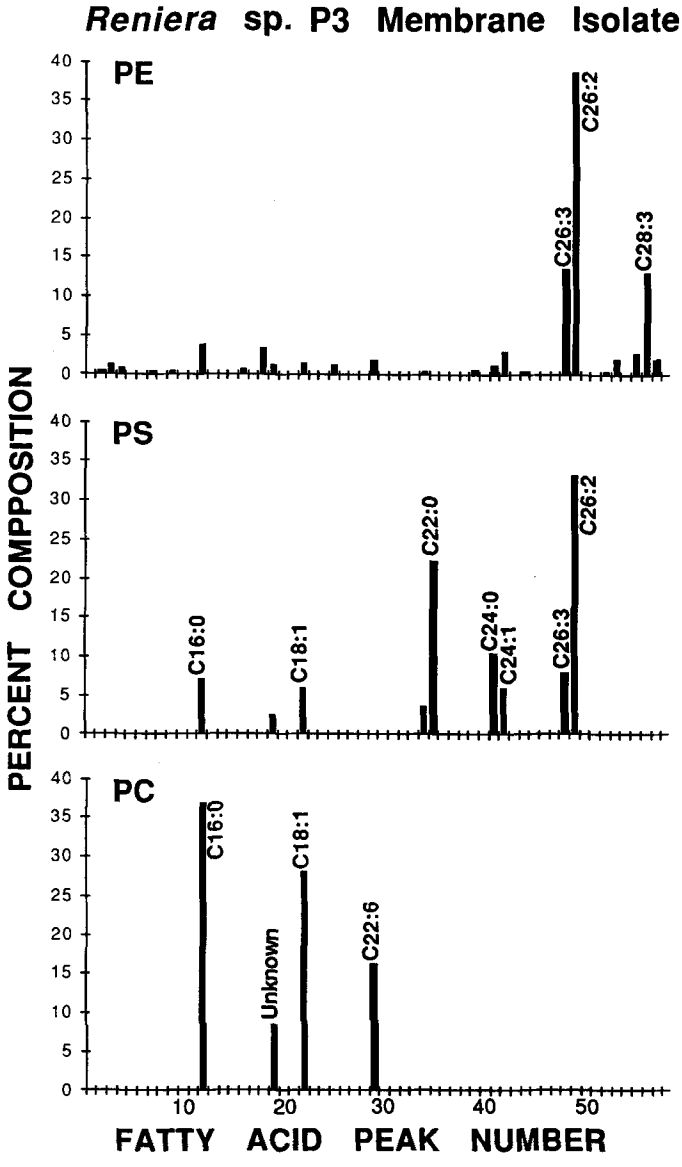

FIG. 5. The proportional distributions of fatty acids in the major phospholipid head groups in a P3 membrane isolate of *Reniera* sp.: phosphatidylethanolamine (PE); phosphatidylserine (PS); phosphatidylcholine (PC). Fatty acids are described in Table 1. Fatty acids that compose ≥5% of the total fatty acids are labeled.

Because we now have examined sponges of both major subclasses of the most prolific sponge class, the Demospongiae, we feel justified in concluding that the occurrence of LCFA in sponge cell membranes is at least characteristic of demosponges, if not all sponges, that contain LCFA. However, the origin of LCFA in sponges has yet to be ascertained because it is not known whether sponges independently synthesize their own short chain fatty acids (i.e., <24 carbon units), whether they incorporate and utilize dietary short chain acids, or whether there is a transfer of short chain acids from symbionts to sponge to be used as such in the sponge or to be further elongated by the sponge as has been demonstrated in our laboratory for two branched LCFA (53,54). Appropriate biosynthetic experiments are underway in our laboratory to answer these outstanding questions.

The presence of LCFA in sponge membranes is incompatible with biomembrane structure and function as it presently is understood (5,11,28,32). The cellular membranes of most living organisms contain phospholipid fatty acids drawn from a very limited pool of structural types. These fatty acids are usually even carbon-numbered, straight chain acids with between 12 and 22 carbon units. Fatty acid chain lengths over 24 carbon units prompt questions about their structural accommodation and molecular interactions within the membrane bilayer.

The accommodation of LCFA within the sponge membrane would be expected to impact membrane structure and organization (e.g., membrane thickness, fatty acid stereochemical orientation, and lipid-lipid and lipid-protein molecular interactions [5]), although there is no information regarding how sponge lipids are organized on a molecular level within membranes or whether they are directly involved in defining membrane functions. There is, however, no superficial morphological distinction between sponge cell membranes and those of other organisms: sponge membranes display typical trilaminate bilayer structure, membrane width, and response to freeze-fracture procedures (1,55, unpublished data from this study).

Questions regarding the structural and physiological implications of siting unusual lipid molecules in sponge cell membranes, the origin of these molecules, and possible reasons why sponges contain these lipids are addressed in the following companion paper to this study (33) where the first direct evidence is provided for the occurrence of unconventional-type sterols in sponge cell membranes.

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REFERENCES

1. Lawson, M.P., Bergquist, P.R., and Cambie, R.C. (1986) *Tissue Cell 18,* 19-26.
2. Lehninger, A.L. (1975) *Biochemistry,* 2nd edn., pp. 280-281 and 288, Worth Publishers, New York.
3. Bergmann, W., and Swift, A.N. (1951) *J. Org. Chem. 16,* 1206-1221.
4. Litchfield, C., Greenberg, A.J., Noto, G., and Morales, R.W. (1976) *Lipids 11,* 567-570.
5. Litchfield, C., and Morales, R.W. (1976) in *Aspects of Sponge Biology* (Harrison, F.W., and Cowden, R.R., eds.) pp. 183-200, Academic Press, New York.
6. Morales, R.W., and Litchfield, C. (1976) *Biochim. Biophys. Acta 431,* 206-216.
7. Dembitskii, B.M. (1981) *Khim. Prir. S. 4,* 511-513.
8. Dembitskii, B.M. (1981) *Khim. Prir. S. 4,* 513-515.
9. Bergquist, P.R., Lawson, M.P., Lavis, A., and Cambie, R.C. (1984) *Biochem. Syst. Ecol. 12,* 63-84.
10. Lawson, M.P., Bergquist, P.R., and Cambie, R.C. (1984) *Biochem. Syst. Ecol. 12,* 375-393.

11. Walkup, R.D., Jamieson, G.C., Ratcliff, M.R., and Djerassi, C. (1981) *Lipids 16,* 631-646.
12. Ayanoglu, E., Walkup, R.D., Sica, D., and Djerassi, C. (1982) *Lipids 17,* 617-625.
13. Ayanoglu, E., Kornprobst, J.M., Aboud-Bichara, A., and Djerassi, C. (1983) *Tetrahedron Lett. 24,* 1111-1114.
14. Ayanoglu, E., Popov, S., Kornprobst, J.M., Aboud-Bichara, A., and Djerassi, C. (1983) *Lipids 18,* 830-836.
15. Wijekoon, W.M.D., Ayanoglu, E., and Djerassi, C. (1984) *Tetrahedron Lett. 25,* 3285-3288.
16. Bergquist, P.R. (1978) *Sponges,* pp. 197-198, Hutchinson, London.
17. Wilkinson, C.R. (1978) *Mar. Biol. 49,* 161-167.
18. Wilkinson, C. (1983) *Science 219,* 410-412.
19. Erwin, J. (1973) in *Lipids and Biomembranes of Eukaryotic Microorganisms,* p. 84, Academic Press, New York.
20. Jain, M.K. (1975) in *Current Topics in Membranes and Transport* (Bronner, F., and Kleinzeller, A., eds.) Vol. 6, pp. 1-57, Academic Press, New York.
21. Demel, R.A., and De Kruyff, B. (1976) *Biochim. Biophys. Acta 457,* 263-276.
22. Nes, W.R. (1984) in *Isoprenoids in Plants, Biochemistry and Function* (Nes, W.R., Fuller, G., and Tsai, L.S., eds.) pp. 325-347, Marcel Dekker Inc., New York.
23. Hadley, N.F. (1985) in *The Adaptive Role of Lipids in Biological Systems,* p. 22, Wiley Press, New York.
24. Wong, M.Y.H., Steck, P.A., and Gray, G.R. (1979) *J. Biol. Chem. 254,* 5734-5740.
25. Minale, L. (1976) *Pure Appl. Chem. 48,* 7-23.
26. Djerassi, C., Theobald, N., Kokke, W.C.M.C., Pak, C., S., and Carlson, M.K. (1979) *Pure Appl. Chem. 51,* 1815-1828.
27. Bergquist, P.R., Hofheinz, W., and Oesterhelt, G. (1978) *Biochem. Syst. Ecol. 8,* 423-453.
28. Carlson, R.M.K., Tarchini, C., and Djerassi, C. (1980) in *Frontiers of Bioorganic Chemistry and Molecular Biology* (Ananchenko, S.N., ed.) pp. 211-224, Pergamon Press, New York.
29. Djerassi, C. (1981) *Pure Appl. Chem. 53,* 873-890.
30. Djerassi, C. (1984) in *Natural Products and Drug Development* (Krogsgaard-Larsen, P., Brogger Christensen, S., and Kofod, H., eds.) pp. 164-178, Munksgaard, Copenhagen.
31. Ikekawa, N. (1985) in *Sterols and Bile Acids* (Danielson, N., and Sjovall, J., eds.) pp. 199-230, Elsevier, Amsterdam.
32. Bloch, K. (1985) in *Biochemistry of Lipids and Membranes* (Vance, D.E., and Vance, J.E., eds.) pp. 1-24, Benjamin/Cummings Publ. Co., Menlo Park, CA.
33. Lawson, M.P., Stoilov, I.L., Thompson, J.E., and Djerassi, C. (1988) *Lipids 23,* 750-754.
34. Hofheinz, W., and Oesterhelt, G. (1979) *Helv. Chim. Acta 62,* 1307-1309.
35. Li, X., and Djerassi, C. (1983) *Tetrahedron Lett. 24,* 665-668.
36. Ha, T.T.B., and Djerassi, C. (1985) *Tetrahedron Lett. 26,* 4031-4034.
37. Ha, T.T.B., Kokke, W.C.M.C., Proudfoot, J.R., and Djerassi, C. (1985) *Steroids 45,* 263-276.
38. Stoilov, I.L., Thompson, J.E., and Djerassi, C. (1986) *Tetrahedron 42,* 4147-4155.
39. Stoilov, I., Back, T.G., Thompson, J.E., and Djerassi, C. (1986) *Tetrahedron 42,* 4156-4160.
40. Spurr, A.R. (1969) *J. Ultrastruct. Res. 26,* 31-43.
41. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
42. Privett, O.S., Dougherty, K.A., Erdahl, W.L., and Stolyhwo, A. (1973) *J. Am. Oil Chem. Soc. 50,* 516-520.
43. Kates, M. (1972) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T.S., and Work, E., eds.), Vol. 3, Part II, pp. 275-600, Elsevier, New York.
44. Portoukalian, J., Meister, R., and Zwingelstein, G. (1978) *J. Chromatogr. 152,* 569-574.
45. Skipski, V.P., and Barclay, M. (1969) in *Methods in Enzymology* (Lowenstein, J.M., ed.), Vol. 14, pp. 530-598, Academic Press, New York.
46. Carreau, J.P., and Dubacq, J.P. (1978) *J. Chromatogr. 151,* 384-390.
47. Christie, W.W. (1982) in *Lipid Analysis,* 2nd edn., p. 61, Pergamon Press, Oxford.
48. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600-608.
49. Andersson, B.A. (1978) *Prog. Chem. Fats other Lipids 16,* 279-308.
50. Lawson, M.P. (1985) *Sponge Lipids,* Ph.D. Thesis, University of Auckland, New Zealand, 185 pp.
51. Jefferts, E., Morales, R.W., and Litchfield, C. (1974) *Lipids 9,* 244-247.
52. Dasgupta, A. (1986) *Marine Phospholipid Studies,* Ph.D. thesis, Stanford University, Stanford, CA, 208 pp.
53. Carballeira, N., Thompson, J.E., Ayanoglu, E., and Djerassi, C. (1986) *J. Org. Chem. 51,* 2751-2756.
54. Raederstorf, D., Shu, A.Y.L., Thompson, J.E., and Djerassi, C. (1987) *J. Org. Chem. 52,* 2337-2346.
55. Lethias, C., Garrone, R., and Mazzorana, M. (1983) *Tissue Cell 15,* 523-535.

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Cell Membrane Localization of Sterols with Conventional and Unusual Side Chains in Two Marine Demosponges[1]

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Subcellular fractionation by differential centrifugation was performed on two previously unstudied marine sponges that predominantly contain either conventional (*Reniera* sp.) or unconventional (*Pseudaxinyssa* sp.) sterols. Direct evidence for the presence of unconventional sterols with C_{24} alkylated side chains in the cellular membranes of *Pseudaxinyssa* sp. is provided, but the presence of unconventional sterols in sponge membranes is shown not to be a universal feature of the Porifera. Possible structural and functional roles of unconventional lipid molecules in sponge cell membranes are discussed. *Lipids 23,* 750–754 (1988).

Marine sponges are distinctive because they commonly contain sterols with unusual patterns of ring unsaturation, methylation and side-chain branching (1–7), although this is not a universal feature of the Porifera because many species contain only conventional, cholesterol-type sterols (4). Indeed, sponges contain the greatest variety of sterols in the animal kingdom (3). The subcellular localization of such unconventional sterols within sponges has not been established, although it has been suggested that such sterols are present in the sponge cell membranes because sterols, notably cholesterol, are principal constituents of cellular membranes in other animals (5–8). Furthermore, it has been proposed that such unconventional sterols may act as stereochemical complements to the long and sometimes multiply branched fatty acids in sponges (5,9,10).

Therefore, the purpose of this study was to establish whether such unconventional sterols, where abundant, can be present in sponge cell membranes in substantial amounts and whether conventional sterols occupy typical membrane positions in the absence of unconventional sterols. The preceding companion study of Lawson et al. (10) is extended here to include the evaluation of cell membrane sterol content for two demosponges with widely divergent sterol compositions.

Pseudaxinyssa sp. presented an excellent example for study because more than 99% of the sterols in this sponge consist of two sterols of unconventional structures (11), each with a multi-branched side chain that results from a complex triple bioalkylation at C_{24} by the sponge (12, 13). These are 24-isopropylcholesterol (2) and its unsaturated counterpart 22-dehydro-24-isopropylcholesterol (1) (Fig. 1A). Furthermore, the minor sterols of this sponge also have novel structures that include cyclopropane (14), tert-butyl and 24-dialkylated sterols (15), and sterols with side chains that exhibit triple and quadruple methylations (16).

Reniera sp. provided an excellent example for comparison as preliminary studies (to be described here) established the sole presence of conventional-type sterols (Fig. 1B). Further, specimens of this sponge that are devoid of microorganism symbionts, as is described in the companion study (10), can be obtained.

EXPERIMENTAL

Sponge samples. Samples of *Reniera* sp. (order Haplosclerida, California Academy of Sciences Museum specimen #063176) and *Pseudaxinyssa* sp. (order Axinellida, Australian Museum specimen #Z4988) were those described in the companion paper (10).

Chemicals and materials. Cholesterol (Sigma Chemical Co., St. Louis, MO) was used for reference in high performance liquid chromatography (HPLC), gas chromatography (GC) and thin layer chromatography (TLC) analyses. TLC plates (silica coated aluminium, Kieselgel 60 F254, 0.2 mm, Merck Chemical Co., Darmstadt, Germany) were washed in diethylether before use. General laboratory reagents were purchased from Sigma or Aldrich (Milwaukee, WI) Chemical Companies, or May and Baker Pty. Ltd. Analytical grade solvents for lipid analysis were distilled before use.

Subcellular fractionation. Whole sponge tissue and tissue isolates were identical to those of the companion study (10) and were generated from the differential centrifugation subcellular fractionation of whole sponge homogenates at 600, 15,000 and 100,000 × g. The subcellular isolates included the pellets P1, P2 and P3 from these respective centrifugations. The cellular and subcellular contents of these pellets were determined by electron microscopy in the companion study (10).

Lipid analysis. Total lipid extracts were subsamples of extracts produced for the companion study (10). Sterols were isolated by column chromatography (Davisil 62 silica gel, elution with hexane/ether [2:1, v/v]). Individual sterols were isolated by reversed-phase HPLC (2 Altex Ultrasphere ODS columns [5 μ, 25 cm × 10 mm i.d.] connected in series; eluent = abs. methanol). Sterols were identified on the basis of relative retention times from GC (Hewlett-Packard model 5790 gas chromatograph; column 2 m × 0.5 mm i.d. OV-17/3% chromasorb; 260 C isothermal), mass spectra (Hewlett Packard 5970 series mass selective detector connected to a 5890A gas chromatograph; 12 m × 0.2 mm i.d. SE-30 fused silica column), and proton nuclear magnetic resonance spectroscopy (^{1}HNMR; 360 MHz Bruker HXS-360). Stereochemical assignments were made from interpretation of ^{1}HNMR spectra and comparison with spectra from earlier studies (17). Relative proportions of sterols were calculated from electronic integration (Hewlett Packard model 3392A integrator) of GC traces.

RESULTS

Pseudsaxinyssa sp. and *Reniera* sp. were found to contrast in the nature and quantity of sterols. As previously

[1]Part 59 of "Sterols in Marine Invertebrates." For Part 58 in this series, see Cho, J.-H., and Djerassi, C. (1987) *J. Org. Chem. 52,* 4517–4521.

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Abbreviations: GC, gas chromatography; HPLC, high performance liquid chromatography; LCFA, long chain fatty acids; TLC, thin layer chromatography.

reported (11), *Pseudaxinyssa* sp. contains two major sterols, 24-isopropylcholesterol (2) and its unsaturated counterpart 22-dehydro,24-isopropylcholesterol (1) (Fig. 1A), that account for more than 99% of the total sterols. In contrast, *Reniera* sp. contains 13 major (>0.5% total) sterols that apparently result from biosynthetically simple methylations at C_{24} and double bond introductions around C_{24} and C_{22} in the side chain of the parent animal sterol cholesterol (5) (Fig. 1B). Stigmasterol (12) and sitosterol (13), which are characteristic sterols of photosynthetic organisms (18), constitute 8.7% of the sterols in *Reniera* sp. (Fig. 1B); such sterols, however, may not be produced de novo by the sponge, but rather may be assimilated from a dietary source such as micro-algae.

The subcellular fractionation method employed produced fractions with the following compositions: P1, partially disrupted cells, collagen, nuclei and other large organelles (>2.8 μ diam.); P2, smaller organelles, and in the case of *Pseudaxinyssa* sp., large numbers of intact cyanobacteria; and P3, membrane vesicles and nonmembrane-bound protein and ribonucleic acid ribosomes (10).

The P3 membrane isolates must be regarded as multimembrane mixtures that could have contained both plasma and organelle membranes from the many cell types present, although the P3 isolates were likely enriched in archaeocyte plasma membrane and endoplasmic reticulum as discussed in the preceding paper (10). The P3 isolates were highly pure in the sense that they were devoid of lipidic, nonmembranous contaminants. Furthermore, there appeared to be little contamination of the P3 membrane isolates by symbiotic microorganisms:

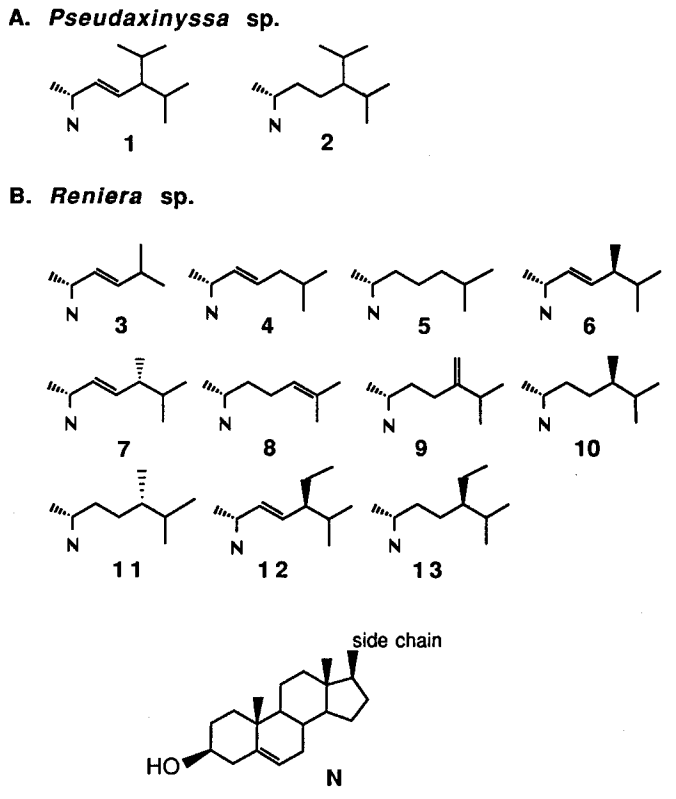

FIG. 1. Major sterols (>0.5%) of: A) *Pseudaxinyssa* sp. and B) *Reniera* sp.

whole *Reniera* sp. specimens contained essentially no microbial symbionts that could have contaminated the P3 isolates, while the cyanobacterial symbionts present in *Pseudaxinyssa* sp. appeared to remain intact during homogenization of the tissue and were largely isolated in the P2 fraction (10). Moreover, it is unlikely that bacteria, including cyanobacteria, would have interfered with sterol analyses as these microorganisms are not known to contain sterols within their plasma membrane (19–22).

The sterol compositions of both *Pseudaxinyssa* sp. and *Reniera* sp. P3 membrane isolates were virtually identical to those sterol compositions observed for whole sponges (Figs. 2 and 3), indicating that any extramembranous, stored sterols were either minor or present in similar proportions to that of the cell membranes. Thus, as has been noted with fatty acid content (10,23), the sterol content of whole sponges provides a valid indication of the sterol content of sponge cell membranes.

The compositions of the P1 and P2 isolates, like the P3 membrane isolate, also reflected the sterol composition of the respective whole sponges, although the mixed natures of these isolates allows no cellular or subcellular conclusions.

DISCUSSION

This study presents the first direct evidence of unconventional sterols occurring in sponge cell membranes, although we also have demonstrated that this is not a universal feature of the Porifera. It is rare for animals to contain sterols other than cholesterol (5) in their cellular membranes (24,25), while plants typically contain sterols with more than eight carbon atoms in the side chain (phytosterols including stigmasterol [12] and sitosterol [13] in higher plants and mycosterols including ergosterol in lower plants) (18).

However, there are a few eucaryotic organisms that substitute the conventional-type sterols of animals and plants with stereochemically similar molecules in their cell membranes (8,20,24,26,27). For example, the freshwater protozoan *Tetrahymena pyriformis* is devoid of membrane sterols and instead contains a sterol-like triterpenoid (tetrahymanol) (28) and in the mycoplasma *Acholeplasma laidlawii,* a low level of cholesterol in the membrane is compensated by a high content of carotenoids (29). In this study, we also see that the unusual membrane sterols 1 and 2 in *Pseudaxinyssa* sp. are stereochemically similar to cholesterol (5), possessing an identical nuclear ring system, a 3β-hydroxy group and a C_{17}-β-side chain alkylation.

Sponge membranes already are known to be unusual in other aspects of their lipid content. More than half of the fatty acids in cell membranes from three demosponges, including the species of *Pseudaxinyssa* and *Reniera* that are the subject of this investigation, consist of long chain C_{24}-C_{30} fatty acids (LCFA), that often exhibit distinctive Δ5,9 unsaturation (10,23), whereas conventional animal cell membranes usually contain fatty acids with chain lengths between C_{14} and C_{22} units (30).

However, there are no clear patterns of lipid content in sponges because unconventional sponge sterols do not always co-occur with LCFA (e.g., *Reniera* sp., this study), and different sponge species produce a heterogeneous and diverse array of fatty acid, sterol and sterol-like

molecules. Still, the most unusual structural features of sponge membrane lipids appear to reside deep within the lipophilic region of the phospholipid bilayer. However, there is indirect evidence that suggests that sponge membrane differences also extend to the hydrophilic regions of the phospholipid bilayer as an unusually high proportion of the amino group-containing phospholipids (phosphatidylethanolamine and phosphatidylserine) are present in sponges (31–33), in contrast with phosphatidylcholine in most animals (30). Further, in dictyoceratid and dendroceratid sponges, which either lack or contain minute amounts of sterols, a membrane role has been suggested for terpenoid sterol analogs (e.g., tetracyclic sesterterpenes) that are present in abundance (23).

The accepted general model for cellular membrane structure envisions a complementary system of sterol

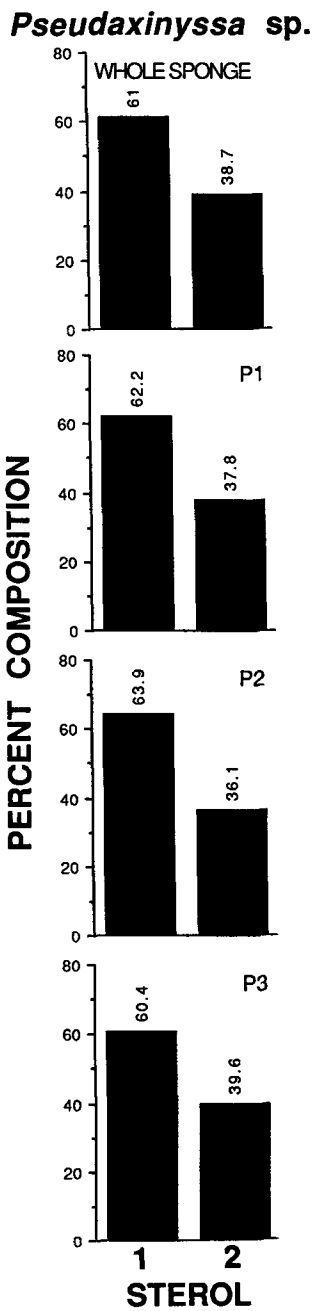

FIG. 2. Percent composition of total sterols present in whole sponge tissue and subcellular tissue isolates from *Pseudaxinyssa* sp. Subcellular fractions were P1, P2 and P3 (membrane isolate), which resulted from differential centrifugation at 600, 15,000 and 100,000 × g, respectively. Sterols are 22-dehydro-24-isopropylcholesterol (*1*) and 24-isopropylcholesterol (*2*) (Fig. 1).

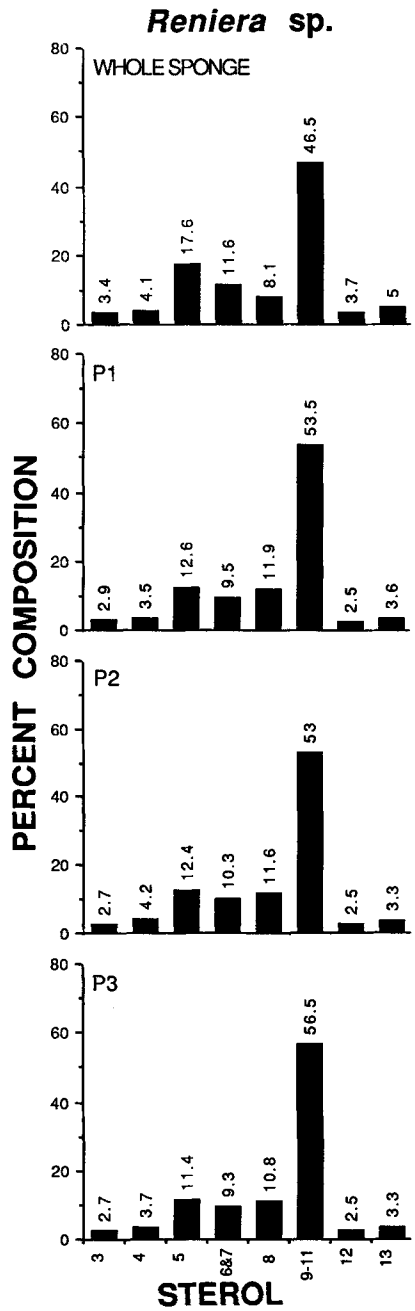

FIG. 3. Percent composition of total sterols present in whole sponge tissue and subcellular fractions of *Reniera* sp. Subcellular fractions, P1, P2 and P3 (membrane isolate), were obtained by differential centrifugation at 600, 15,000 and 100,000 × g, respectively. Sterols are 22-dehydro-24-norcholesterol (*3*), 22-dehydrocholesterol (*4*), cholesterol (*5*), crinosterol (*6*), brassicasterol (*7*), desmosterol (*8*), 24-methylenecholesterol (*9*), campesterol (*10*), dihydrobrassicasterol (*11*), stigmasterol (*12*), and sitosterol (*13*) (Fig. 1).

molecules intercalated between the fatty acyl chains of a phospholipid bilayer (34). In the same manner, our laboratory (5,9) has proposed that some unique structural features of sterols and fatty acids in sponges structurally complement each other in the cell membranes, although such interaction has yet to be demonstrated. For example, Walkup et al. (9) hypothesized that aplysterol, a C_{24} and C_{26} side chain alkylated sterol, might preferentially pair with monomethyl-branched or $\omega 7$ unsaturated fatty acid chains in the sponge *Aplysina fistularis;* however, more recent model membrane studies in our laboratory (35) with this sponge do not support such a hypothesis. It is likely that sponge membrane proteins contribute in an essential manner and the nature of these macromolecules is now under examination in our laboratory.

In addition to structural roles, membrane sterols also may modulate a variety of physiological regulatory processes (25,36), although it is unknown whether unconventional sponge sterols serve any such roles. It is tempting to speculate that the presence of unconventional sterols in sponges is related to some biological or ecological situation unique to sponges. For example, sponges are characterized by a unique cell-cell recognition system dependent on membrane-bound, proteinaceous aggregation factors (37). In addition, many sponges also contain large quantities of species-specific bacterial symbionts (38). Furthermore, as a group, sponges are capable of occupying virtually any aquatic environment, possibly varying membrane LCFA content within species-specific ranges according to environmental temperature (39).

Although sponges can be regarded as a very successful group by evolutionary terms, as they have been present at least since the mid-Cambrian, and there are over 5,000 extant species (37), the production of so-called primitive lipid molecules by sponges (e.g., the anaerobically formed terpenes and β-face demethylated sterols), together with the failure of the Porifera to give rise to evolutionarily more advanced lines, has resulted in sponges being regarded as a dead end branch of the phylogenetic tree (8). Still, the question remains unanswered why the Porifera evolved methods to produce, or assimilate from dietary sources, and perpetrate this range of complex molecules when more advanced animals rely on cholesterol and a narrower array of simpler fatty acids with chain lengths between C_{14} and C_{22} in their cell membranes.

The production of such unusual lipids by sponges may either be due to chance mutations in biosynthetic routes that did not affect species fitness or due to selection for particular cell membrane or physiological molecules (8). The first explanation might apply if the bulk structures of the lipid molecules fulfill a purely structural role in the cell membrane. The latter explanation might, however, apply if: 1) minor differences in the parent molecule's structure allowed improved structural (e.g., conformational alignment of membrane molecules) or functional membrane organization and/or 2) if modified sterols could serve some important nonmembrane functional role.

Of equal interest is whether sponges synthesize unusual membrane sterols and other lipids de novo. In some sponges, it has been observed that sterols cannot originate from de novo biosynthesis, but arise by modification of dietary sterols (40-43). This supports Bloch's (25) suggestion that marine invertebrates, regardless of whether they are sterol auxotrophs, appear to discriminate much less against dietary sterols with the result that their tissue sterol compositions show varying degrees of complexity. Goad (44) and Djerassi (6) have remarked that unusual sterols in sponges may be a result of sponge interaction with symbionts.

None of these questions can be answered, however, until sponge cellular and subcellular membrane lipid compositions are more carefully characterized as 1) sterol content is expected to vary widely from species to species and from one cell or intracellular membrane system to another based on studies of mammalian cells (45), and 2) different sponge cells and their subcellular membrane systems fulfill different functions (37). Studies that seek to characterize the sterol distributions of particular sponge cell types and their subcellular membrane systems are underway in our laboratory. In addition, we have undertaken studies with synthetic vesicles consisting of pure long chain fatty acid phospholipids to describe more fully the limits to structural and functional interactions and to determine the physical properties of unusual lipids in sponge membranes (35).

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REFERENCES

1. Minale, L. (1976) *Pure Appl. Chem. 48,* 7-23.
2. Ikekawa, N. (1985) in *Sterols and Bile Acids* (Danielson, N., and Sjovall, J., eds.) pp. 199-230, Elsevier, Amsterdam.
3. Djerassi, C., Theobald, N., Kokke, W.C.M.C., Pak, C.S., and Carlson, R.M.K. (1979) *Pure Appl. Chem. 51,* 1815-1828.
4. Bergquist, P.R., Hofheinz, W., and Oesterhelt, G. (1978) *Biochem. Syst. Ecol. 8,* 423-453.
5. Carlson, R.M.K., Tarchini, C., and Djerassi, C. (1980) in *(IUPAC) Frontiers of Bioorganic Chemistry and Molecular Biology* (Anan-chenko, S.N., ed.) pp. 211-224, Pergamon Press, Oxford.
6. Djerassi, C. (1981) *Pure Appl. Chem. 53,* 873-890.
7. Djerassi, C. (1984) in *Natural Products and Drug Development* (Krogsgaard-Larsen, P., Brogger-Christensen, S., and Kofod, H., eds.) pp. 164-178, Munksgaard, Copenhagen.
8. Bloch, K.E. (1983) *Cr. Rev. Biochem. 14,* 47-92.
9. Walkup, R.D., Jamieson, G.C., Ratcliff, M.R., and Djerassi, C. (1981) *Lipids 16,* 631-646.
10. Lawson, M.P., Thompson, J.E., and Djerassi, C. (1988) *Lipids 23,* 741-749.
11. Hofheinz, W., and Oesterhelt, G. (1979) *Helv. Chim. Acta 62,* 1307-1309.
12. Stoilov, I.L., Thompson, J.E., and Djerassi, C. (1986) *Tetrahedron 42,* 4147-4155.
13. Stoilov, I.L., Back, T.G., Thompson, J.E., and Djerassi, C. (1986) *Tetrahedron 42,* 4156-4160.
14. Ha, T.B.T., and Djerassi, C. (1985) *Tetrahedron Lett. 26,* 4031-4034.
15. Li, X., and Djerassi, C. (1983) *Tetrahedron Lett. 24,* 665-668.
16. Ha, T.B.T., Kokke, W.C.W.C., Proudfoot, J.R., Djerassi, C., and Thompson, J.E. (1985) *Steroids 45,* 263-276.
17. Kokke, W.C.M.C., Pak, C.S., Fenical, W., and Djerassi, C. (1979) *Helv. Chim. Acta 62,* 1310-1318.
18. McKersie, B.D., and Thompson, J.E. (1979) *Plant Physiol. 63,* 802-805.
19. Jain, M.K. (1975) in *Current Topics in Membranes and Transport* (Bronner, F., and Kleinzeller, A., eds.) Vol. 6, pp. 1-57, Academic Press, New York.

20. Demel, R.A., and DeKruyff, B. (1976) *Biochim. Biophys. Acta 457,* 109-132.
21. Nes, W.R. (1984) in *Isoprenoids in Plants, Biochemistry and Function* (Nes, W.R., Fuller, G., and Tsai, L.S., eds.) pp. 325-347, Marcel Dekker Inc., New York.
22. Hadley, N.F. (1985) *The Adaptive Role of Lipids in Biological Systems,* p. 22, Wiley Press, New York.
23. Lawson, M.P., Bergquist, P.R., and Cambie, R.C. (1986) *Tissue Cell 18,* 19-26.
24. Nes, W.R. (1974) *Lipids 9,* 596-612.
25. Bloch, K.E. (1985) in *Biochemistry of Lipids and Membranes* (Vance, D.E., and Vance, J.E., eds.) pp. 1-24, Benjamin/Cummings Publ. Co., Menlo Park, CA.
26. Smith, P.F. (1971) *The Biology of Mycoplasmas,* Academic Press, New York.
27. Rohmer, M., Bouvier, P., and Ourisson, G. (1979) *Proc. Natl. Acad. Sci. USA 76,* 847-851.
28. Thompson, G.A. Jr., Bambury, R.J., and Nozawa, Y. (1972) *Biochim. Biophys. Acta 260,* 630-638.
29. Rottem, S., and Markowitz, O. (1979) *J. Bacteriol. 40,* 944-948.
30. Lehninger, A.L. (1975) *Biochemistry,* 2nd edn., pp. 280-281 and 288, Worth Publishers, New York.
31. Ayanoglu, E., Walkup, R.D., Sica, D., and Djerassi, C. (1982) *Lipids 17,* 617-625.
32. Ayanoglu, E., Popov, S., Kornprobst, J.M., Aboud-Bichara, A., and Djerassi, C. (1983) *Lipids 18,* 830-836.
33. Dasgupta, A., Ayanoglu, E., Wegmann-Szente, A., Tomer, K., and Djerassi, C. (1986) *Lipids 41,* 335-347.
34. Rothman, J.E., and Engelman, D.M. (1972) *Nature 237,* 42-44.
35. Ayanoglu, E., Duzgunes, N., Wijekoon, W.M.D., and Djerassi, C. (1986) *Biochim. Biophys. Acta 863,* 110-114.
36. Morris, R.J., and Culkin, F. (1977) *Oceanogr. Mar. Biol. Ann. Rev. 15,* 73-102.
37. Bergquist, P.R. (1978) *Sponges,* pp. 9 and 52-78, Hutchinson, London.
38. Wilkinson, C.R., Nowak, M., Austin, B., and Colewell, R.R. (1985) *Microb. Ecol. 7,* 13-21.
39. Lawson, M.P. (1985) *Sponge Lipids,* Ph.D. Thesis, University of Auckland, New Zealand, 185 pp.
40. DeRosa, M., Minale, L., and Sodano, G. (1975) *Experientia 31,* 408-410.
41. Catalan, C.A.N., Thompson, J.E., Kokke, W.C.M.C., and Djerassi, C. (1985) *Tetrahedron 41,* 1073-1084.
42. Stoilov, I.L., Thompson, J.E., Cho, J.-H., and Djerassi, C. (1986) *J. Am. Chem. Soc. 108,* 8235-8241.
43. Stoilov, I.L., Bladocha-Moreau, M., Thompson, J.E., and Djerassi, C. (1987) *Tetrahedron 43,* 2213-2222.
44. Goad, L.J. (1978) in *Marine Natural Products* (Scheuer, P.J., ed.) Vol. 2, pp. 76-173, Academic Press, New York.
45. Lange, Y., and Ramos, B.V. (1983) *J. Biol. Chem. 258,* 15130-15134.

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Effects of 2-(2,4-Dimethylphenyl)indan-1,3-dione on Serum Lipoprotein and Lipid Metabolism of Rodents

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2-(2,4-Dimethylphenyl)indan-1,3-dione was shown to be a potent hypolipidemic agent in rodents, lowering significantly both serum cholesterol and triglyceride levels at 20 mg/kg/day. The agent in vivo inhibited the enzymatic activities of ATP-dependent citrate lyase, acetyl-CoA synthetase, cholesterol-7-α-hydroxylase, acyl-CoA cholesterol acyl transferase, *sn*-glycerol-3-phosphate acyl transferase and phosphatidylate phosphohydrolase. Tissue lipid levels of liver and small intestine also were reduced by the agent. The rat serum lipoprotein lipid content was modulated by the drug, which should be favorable for the removable of cholesterol from peripheral tissue for conduction to the liver for clearance from the body. Low density lipoprotein (LDL) cholesterol levels were reduced after treatment, which suggests that the agent potentially reduces deposition of cholesterol in plaques. If chemotherapy for atherosclerosis is to be successful, then the high density lipoprotein (HDL) cholesterol level needs to be elevated more than 16% to 25%, the level produced by current hypolipidemic agents. 2-(2,4-Dimethylphenyl)-indan-1,3-dione offers a 75% increase in HDL cholesterol levels and a 30% reduction of LDL cholesterol levels with a suppression of de novo synthesis of lipids and a reduction of tissue cholesterol deposition.
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The hypolipidemic activity of a series of N-substituted cyclic imides (1–4), i.e., phthalimide, 1,8-naphthalimide, diphenimide, glutarimide and saccharin, has been well-established in rodents. Further structure activity studies have demonstrated that 2-substituted indan-1,3-dione possesses potent hypolipidemic activity in mice (5). Of this series, the 2-(2,4-dimethyl-phenyl)indan-1,3-dione derivatives proved to be one of the more potent compounds of the series lowering serum cholesterol levels by 46% and serum triglyceride levels by 42% after 16 days dosing at 20 mg/kg/day i.p. to mice. The N-substituted cyclic imides were not HMG-CoA reductase inhibitors, rather they inhibited the enzymatic step in the early synthesis of cholesterol. Triglyceride synthesis was inhibited at the rate-limiting enzyme of the de novo synthetic pathway, i.e., *sn*-glycerol-3-phosphate acyl transferase and phosphatidylate phosphohydrolase (5). At this time, a more in-depth study was undertaken to examine the effects of this agent on lipid metabolism in rodents to establish if the agent is feasible for clinical use as a hypolipidemic agent against hyperlipidemic disease states and atherosclerosis. Specifically, the purpose of the investigation was to determine if 2-(2,4-dimethylphenyl)-indan-1,3-dione raised high density lipoprotein (HDL) cholesterol levels and lowered low density lipoprotein (LDL) cholesterol levels, a ratio that supposedly protects man against myocardial infarctions (6). Previous studies have shown that N-substituted cyclic imides did not modulate this ratio rapidly enough (1) to be effective in a clinical situation to treat atherosclerotic patients.

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Abbreviations: HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Sources of compounds. 2-(2,4-Methylphenyl)indan-1,3-dione was synthesized by the procedure previously outlined (5), and the physical and chemical characteristics were consistent with those reported. Isotopes were purchased from New England Nuclear (Boston, MA). Substrates and cofactors were obtained from Sigma Chemical Co. (St. Louis, MO). Clofibrate was obtained from Ayerst Laboratories (New York, NY).

Antihyperlipidemic screens in normal rodents. Compounds were suspended in 1% carboxy methyl cellulose-water, homogenized and administered to CF_1 male mice (~25 g) intraperitoneally for 16 days. On days 9 and 16, blood was obtained by tail vein bleeding and the serum separated by centrifugation for three min. Sprague-Dawley rats (~300 g) were administered compounds at 20 mg/kg/day orally by intubation needle for 14 days. Blood samples were collected by tail vein bleeding on days 9 and 14. Control animals were administered 0.2 ml of 1% carboxy methyl cellulose as the vehicle and bled on the same days. All blood samples were collected between 8–9 a.m. Nonhemolyzed samples were used for the assays. The serum cholesterol levels were determined by a modification of the Liebermann-Burchard reaction (7). The triglyceride content was determined using the Bio Dynamic/bmc Triglyceride Kit, Boehringer Mannheim (Indianapolis, IN). Serum was also collected on day 16 for mice and on day 14 for rats.

Testing in hyperlipidemic mice. CF_1 male mice (~25 g) were placed on a commercial diet (U.S. Biochemical Corporation Basal Atherogenic diet) that contained butterfat (400 g), celufil (cellulose) (60 g), cholesterol (53 g), choline dihydrogen citrate (4 g), Wesson oil (40 g), sodium cholate (20 g), sucrose (223 g), vitamin-free casein (200 g) and total vitamin supplement for 10 days. After the cholesterol and triglyceride levels were assayed and observed to be elevated, the mice were administered test drugs at 20 mg/kg/day intraperitoneally for an additional 14-day period. Serum cholesterol and triglyceride levels were measured after 14 days of administration of the drugs.

Enzymatic studies. In vitro enzymatic studies were determined using 10% homogenates in 0.25 M sucrose + 1 mM EDTA, pH 7.2 of CF_1 mouse liver with 50–200 μM of test drug. In vivo enzymatic studies were determined using 10% homogenates of liver from CF_1 male mice obtained after administering the agents for 16 days at a dose of 20 mg/kg/day intraperitoneally. Acetyl-CoA synthetase (8) and adenosine triphosphate-dependent citrate lyase (9) activities were determined spectrophotometrically at 540 nm as the hydroxamate of acetyl-CoA formed after 30 min at 37 C. Mitochondrial citrate exchange was determined by the procedure of Robinson et al. (10,11) using

^{14}C-sodium bicarbonate (41 mCi/mmol) incorporated into mitochondria. ^{14}C-citrate was determined after incubating the mitochondrial fraction, which was loaded with labeled citrate and test drugs for 10 min. Then the radioactivity was measured in the mitochondrial and supernatant fractions of Fisher Scintiverse fluid using a Packard Scintillation counter and expressed as a percentage of the total counts. Cholesterol 7α-hydroxylase activity was determined by the method of Shefer et al. (12) using [1,2-^{3}H(N)]-cholesterol (54.8 mCi/mmol). Acyl cholesterol acyl transferase activity was determined using ^{14}C-oleic acid (50 mCi/mmol). Oleic acid was separated from cholesteryl esters on thin layer chromatography (TLC) silica gel eluted with ether (13). 3-Hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) activity was measured using [1-^{14}C]acetyl-CoA (56 mCi/mmol) and a post-mitochondrial supernatant (9000 g $\times$ 20 min) incubated for 60 min at 37 C (14). The digitonide derivative of cholesterol was isolated and counted (15). Acetyl-CoA carboxylase activity was measured by the method of Greenspan and Lowenstein (16). Initially, the enzyme had to be polymerized for 30 min at 37 C, and then the assay mixture containing sodium ^{14}C-bicarbonate (41.0 mCi/mmol) was added and incubated for 30 min at 37 C with test drugs. *sn*-Glycerol-3-phosphate acyl transferase activity was determined with glycerol-3-phosphate [L-2-^{3}H(N)] (7.1 Ci/mmol) and the microsomal fraction of the liver (17). The reaction was terminated after 20 min, and the lipids were extracted with chloroform/methanol (2:1) containing 1% conc. HCl and counted. Phosphatidylate phosphohydrolase activity was measured as the inorganic phosphate released after 30 min from phosphatidic acid by the method of Mavis et al. (18). The released inorganic phosphate after development with ascorbic acid and ammonium molybdate was determined at 820 nm. Hepatic heparin-induced lipoprotein lipase activity was determined using the method of Chait et al. (19).

Liver lipid extraction. In CF_1 male mice that had been administered test drugs for 16 days, the livers were removed, and in Sprague-Dawley rats treated orally with the test drug 14 days, the liver, small intestine and 24-hr fecal samples were collected. A 10% homogenate in 0.25 M sucrose + 1 mM (ethylenedinitrilo)tetraacetic acid was prepared for each tissue. An aliquot (2 ml) of the homogenate was extracted by the methods of Folch et al. (20) and Bligh and Dyer (21), and the number of mg of lipid weighed. The lipid was taken up in methylene chloride and the cholesterol level (7), triglyceride levels (Bio-Dynamics/bmc Triglyceride Kit), neutral lipid content (22) and phospholipid content (23) were determined. Protein was determined by the method of Lowry et al. (24).

^{3}H-cholesterol distribution in rats. Sprague-Dawley male rats (~350 g) were administered the test compound for 14 days orally at 20 mg/kg/day. On day 13, 10 μCi of ^{3}H-cholesterol was administered orally to rats, and feces were collected over the next 24 hr. Twenty-four hr after cholesterol administration, the major organs were excised and samples of blood, chyme and urine were obtained. Homogenates (10%) were prepared of the tissues that were combusted (Packard Tissue Oxidizer) and counted (25). Some tissue samples were plated on filter paper (Whatman #1) dried and digested for 24 hr in 30% KOH at 40 C and counted. Results were expressed as dpm per total organ.

Plasma lipoprotein fractions. Sprague-Dawley male rats (~350 g) were administered the test compound at 20 mg/kg/day orally for 14 days. On day 14, blood was collected from the abdominal vein. Serum was separated from whole blood by centrifugation at 3500 rpm. Aliquots (9 ml) were separated by ultracentrifugation, according to the methods of Havel et al. (26) and Hatch and Lees (27) as modified for normal rats (28), into the chylomicrons, very low density lipoproteins (VLDL), HDL and LDL. Each of the fractions was analyzed for cholesterol, triglyceride, neutral lipids, phospholipids and protein levels.

Acute toxicity. The LD_{50} values were determined in CF_1 mice (~25 g) by administering test drug intraperitoneally from 100 mg/kg to 1 g/kg as a single dose. The number of deaths were recorded over a seven-day period for each group.

Animal weights and food intake. Periodic animal weights were obtained during the experiment and expressed as a percentage of the rat's weight on day 0. After dosing for 14 days with the test drug at 20 mg/kg/day, selected organs were excised, trimmed of fat and weighed. The food consumption was determined daily and expressed as gm/day/rat.

RESULTS

2-(2,4-Methylphenyl)indan-1,3-dione was shown to be a potent hypolipidemic agent in rodents. A dose response study in mice from 5 to 40 mg/kg/day i.p. demonstrated that the maximum lowering of serum cholesterol levels of 46% and the maximum lowering of serum triglyceride levels of 42% was achieved at 20 mg/kg/day i.p. for 16 days. At this same dose administered orally to rats, serum cholesterol levels were reduced 40% and serum triglyceride levels were reduced 48% after 14 days (Table 1). The agent was also found to be effective in diet-induced hyperlipidemic states in mice, where the serum cholesterol level was reduced 40% and serum triglyceride levels were reduced 25% at 20 mg/kg/day for 14 days (Table 1).

2-(2,4-Dimethylphenyl)indan-1,3-dione was shown to inhibit in vitro mitochondrial citrate exchange and ATP-dependent citrate lyase greater than 25% (Table 2). Acetyl-CoA synthetase activity was inhibited greater than 50% at all three concentrations of drug. HMG-CoA reductase, acetyl-CoA, carboxylase and heparin-induced lipoprotein lipase activities were not affected by the presence of the drug. Cholesterol-7α-hydroxylase and acyl cholesterol acyl transferase activities were inhibited in a concentration-dependent manner, with the latter enzyme being inhibited 81% at 200 μM. *sn*-Glycerol-3-phosphate acyl transferase and phosphatidylate phosphohydrolase activities were inhibited in a concentration-dependent manner with 200 μM, resulting in greater than 50% inhibition.

The in vivo effects of the agent on the identical enzyme activities demonstrated that the agent was a potent inhibitor of acetyl-CoA synthetase activity, i.e., greater than 50%, and ATP-dependent citrate lyase activity, i.e., greater than 60% (Table 3). HMG-CoA reductase activity was elevated for all four doses of the drug. Cholesterol-7α-hydroxylase activity was suppressed 44% at 40 mg/kg/day. Acetyl-CoA cholesterol acyl transferase activity

TABLE 1

Effects of 2-(2,4-Methylphenyl)indanone on Serum Lipid of CF_1 Mice and Sprague-Dawley Rats

		Mg % (X ± SD)			
		Serum cholesterol		Serum triglyceride	
		Day 9	Day 16	Day 9	Day 16
CF_1 mice (N = 6)	Normal				
Control	1% CMC	128 ± 6	130 ± 5	136 ± 6	137 ± 7
5 mg/kg		102 ± 5*	81 ± 5*	101 ± 7*	57 ± 4*
10 mg/kg		88 ± 5*	78 ± 6*	83 ± 6*	79 ± 4*
20 mg/kg		79 ± 6*	70 ± 4*	95 ± 5*	93 ± 5*
40 mg/kg		90 ± 5*	71 ± 4*	67 ± 5*	101 ± 4*
		Serum cholesterol		Serum triglyceride	
		Day 7	Day 14	Day 7	Day 14
Sprague-Dawley rats (N = 6)					
Control	1% CMC	75 ± 7	78 ± 5	109 ± 5	110 ± 5
Treated		48 ± 9*	47 ± 3*	71 ± 6*	57 ± 5*
		Serum cholesterol		Serum triglyceride	
Atherogenic diet-induced CF_1 mice					
Control diet		354 ± 25		367 ± 24	
Treated 20 mg/kg/day		212 ± 4*		275 ± 23*	

*P < 0.001.

TABLE 2

Effects in vitro of 2-(2,4-Methylphenyl)indan-1,3-dione on Enzyme Activities Involved in Hepatic Lipid Metabolism (N = 6)

	Compound (percent of control [X ± SD])			
Enzyme	Control	50 μM	100 μM	200 μM
Mitochondrial citrate exchange	100 ± 5[a]	75 ± 2*	69 ± 4*	72 ± 6*
ATP-dependent citrate lipase	100 ± 5[b]	62 ± 4*	69 ± 3*	64 ± 5*
Acetyl-CoA synthetase	100 ± 7[c]	44 ± 3	49 ± 6*	44 ± 4*
HMG-CoA reductase	100 ± 6[d]	107 ± 7	105 ± 3	118 ± 7
Cholesterol-7α-hydroxylase	100 ± 4[e]	86 ± 6	74 ± 6*	67 ± 3*
Acyl cholesterol acyl transferase	100 ± 5[f]	58 ± 5*	44 ± 4*	19 ± 2*
Acetyl-CoA carboxylase	100 ± 6[g]	94 ± 5	99 ± 5	99 ± 6
sn-Glycerol-3-phosphate acyl transferase	100 ± 6[h]	81 ± 8	53 ± 8*	49 ± 6*
Phosphatidylate phosphohydrolase	100 ± 6[i]	52 ± 5*	42 ± 6*	41 ± 6*
Heparin-induced lipoprotein lipase	100 ± 5[j]	82 ± 7	88 ± 6	85 ± 78

[a]30.8 mg exchange of mitochondrial citrate.
[b]30.5 mg of citrate lipase/g of wet tissue/30 min.
[c]28.5 mg acetyl-CoA formed/g wet tissue/30 min.
[d]38490 dpm cholesterol formed/g wet tissues/60 min.
[e]224000 dpm/mg of microsomal protein/20 min.
[f]4800 dpm/mg of microsomal protein/30 min.
[g]3201 dpm/g wet tissue/30 min.
[h]537800 dpm/g wet tissue/30 min.
[i]16.7 μg Pi/g wet tissue/15 min.
[j]278500 dpm/g wet tissue/60 min.
*P < 0.001.

TABLE 3

Effects of 2-(2,4-Methylphenyl)indanone on CF_1 Mouse Liver de novo Lipid Synthesis Enzyme Activities After 16 Days Drug Administration i.p. (N = 6)

	Percent of control (X ± SD)				
	ATP-dependent citrate lyase	Acetyl-CoA synthetase	HMG-CoA reductase	Cholesterol 7α-hydroxylase	Acyl-CoA cholesterol acyl transferase
Control 1% CMC	100 ± 7	100 ± 7	100 ± 6	100 ± 5	100 ± 4
Treated					
5 mg/kg/day	67 ± 7*	31 ± 3*	181 ± 8	98 ± 5	76 ± 6*
10 mg/kg/day	47 ± 3*	21 ± 3*	215 ± 8*	72 ± 5*	57 ± 6*
20 mg/kg/day	56 ± 3*	30 ± 3*	221 ± 8*	73 ± 4*	8 ± 2*
40 mg/kg/day	48 ± 3*	28 ± 3*	177 ± 10*	56 ± 7*	39 ± 3*
	Acetyl-CoA carboxylase	*sn*-Glycerol-3 phosphate acyl transferase	Phosphatidylate phosphohydrolase	Heparin lipoprotein lipase	
Control 1% CMC	100 ± 7	100 ± 4	100 ± 6	100 ± 7	
Treated					
5 mg/kg/day	107 ± 8	34 ± 4*	92 ± 6	89 ± 6	
10 mg/kg/day	156 ± 7*	27 ± 4*	76 ± 5*	68 ± 6*	
20 mg/kg/day	150 ± 7*	105 ± 6	59 ± 5*	75 ± 5*	
40 mg/kg/day	125 ± 8	44 ± 8*	35 ± 5*	106 ± 6*	

*P < 0.001.

TABLE 4

Effects of 2-(2,4-Methylphenyl)indanone on CF_1 Mouse Liver Lipids After 16 Days Administration i.p. (N = 6)

	Mg/gm wet tissue					
	mg of lipid extracted	Cholesterol	Triglyceride	Neutral lipids	Phospholipids	Protein
Control	59.2 ± 2.9	12.24 ± 0.73	4.77 ± 0.29	28.35 ± 1.98	4.39 ± 0.35	4.5 ± 0.3*
Treated						
5 mg/kg/day	35.8 ± 2.8*	10.04 ± 0.49*	2.90 ± 0.22*	26.08 ± 2.27	4.03 ± 0.30	5.3 ± 0.2*
10 mg/kg/day	35.2 ± 3.0*	8.69 ± 0.71*	2.38 ± 0.21*	25.51 ± 2.25	4.00 ± 0.29	5.6 ± 0.3*
20 mg/kg/day	33.7 ± 2.7*	8.56 ± 0.61*	2.06 ± 0.19*	28.07 ± 2.53	3.95 ± 0.21	5.0 ± 0.3
40 mg/kg/day	24.8 ± 2.3*	9.79 ± 0.49*	2.48 ± 0.30*	26.37 ± 2.41	3.91 ± 0.37	5.22 ± 0.2

*P < 0.001.

was reduced 92% at 20 mg/kg/day. Acetyl-CoA carboxylase activity was also elevated by the drug from 10 to 40 mg/kg/day. *sn*-Glycerol-3-phosphate acyl transferase activity was reduced greater than 65% at 5 and 10 mg/kg of drug, but elevated 130% at 40 mg/kg/day. Phosphatidylate phosphohydrolase activity was suppressed by the drug in a dose-dependent manner with 65% inhibition at 40 mg/kg/day. Heparin-induced hepatic lipoprotein lipase activity was reduced 32% at 5 mg/kg and 25% at 20 mg/kg/day of drug.

When the lipid content in tissues was evaluated, mice dosed for 16 days showed a reduction in lipid content at all four doses employed (Table 4). Cholesterol and triglyceride contents were reduced at all doses. Neutral lipids demonstrated essentially no change after drug administration and phospholipid content was unchanged at all doses in mice (Table 4). Similar studies in rats treated with the agent at 20 mg/kg/day orally showed that lipid content was reduced in the liver and small intestine (Table 5). Cholesterol content was reduced 18% in the liver and 29% in the small intestine. Triglyceride content was reduced 49% in the liver and 28% in the small intestine of treated rats. Neutral lipids were suppressed 15% in the small intestine but not in the liver. Phospholipids were lowered in the small intestine by 28%. Protein content of the liver was reduced 13%. Fecal samples from treated rats demonstrated an 18% increase in cholesterol content.

Serum lipoproteins lipid content also was examined after treating rats for 14 days at 20 mg/kg/day. Cholesterol content was reduced 30% in the LDL fraction, whereas cholesterol content was elevated 75% in the HDL fraction and 114% in the chylomicron fraction. Triglyceride content was reduced 44% in the HDL fraction. Neutral lipid content was elevated 127% in the LDL and reduced 26% in the HDL fraction. Phospholipid content

TABLE 5

Effects of 2-(2,4-Methylphenyl)indanone on Sprague-Dawley Male Rats Lipid Content of Liver, Small Intestine, Feces and Serum Lipoproteins After 14 Days Administration at 20 mg/kg/day Orally (N = 6)

	mg of lipid extract	Cholesterol	Triglyceride	Neutral lipid	Phospholipid	Protein
			Mg of gram wet tissue			
Liver						
Control	58.5 ± 3.5	24.03 ± 1.44	6.37 ± 0.43	44.11 ± 3.10	7.19 ± 0.36	4.5 ± 0.26
Treated	31.0 ± 29*	19.7 ± 1.39*	3.25 ± 0.30*	41.61 ± 2.94	7.75 ± 0.52	3.93 ± 0.24
Small intestine						
Control	45.5 ± 2.73	7.82 ± 0.45	1.12 ± 0.05	6.98 ± 0.41	2.06 ± 0.16	4.20 ± 0.25
Treated	36.4 ± 2.26	5.54 ± 0.38*	0.80 ± 0.03	5.93 ± 0.57	1.48 ± 0.15	4.45 ± 0.28
Fecal						
Control	8.01 ± 0.48	2.85 ± 1.71	1.86 ± 0.07	3.39 ± 0.27	1.39 ± 0.10	6.99 ± 0.35
Treated	8.49 ± 0.56	3.35 ± 0.85	1.43 ± 0.13	3.11 ± 0.24	1.19 ± 0.08	6.92 ± 0.41
Serum lipoproteins			μg/ml serum			
Chylomicron						
Control	337 ± 23	420 ± 25	67 ± 4	149 ± 7	184 ± 11	
Treated	721 ± 20*	394 ± 34	89 ± 4	132 ± 8	166 ± 9	
VLDL						
Control	190 ± 11	22 ± 2	98 ± 6	26 ± 2	50 ± 3	
Treated	202 ± 12	21 ± 2	83 ± 8	15 ± 4*	49 ± 3	
LDL						
Control	210 ± 13	45 ± 3	10 ± 1	41 ± 2	122 ± 7	
Treated	147 ± 10*	43 ± 3	22 ± 1*	25 ± 3*	74 ± 6*	
HDL						
Control	544 ± 38	27 ± 1	620 ± 50	153 ± 14	65 ± 5	
Treated	952 ± 22*	15 ± 2*	459 ± 31*	155 ± 12	63 ± 3	

*P < 0.001.

was reduced 43% in the VLDL and 37% in the LDL fraction. The protein content of the LDL fraction was reduced 39% (Table 5).

^{3}H-Cholesterol distribution studies showed that after drug administration for 14 days to rats at 20 mg/kg/day orally, the tissue in tested animals demonstrated a slight increase in radioactivity content. The lungs, liver, reproductive organs, stomach, spleen and kidney demonstrated reductions in radioactive content after 14 days of drug administration. Elevations in radioactive content were observed in the small intestine (67%), large intestine (21%), feces (33%) and chyme (4%) (Table 6).

The organ weights of the treated animals demonstrated small reductions for most of the organs, compared with the control animals' weights. The adrenal weights were reduced in the treated animals, indicating no hypertrophy due to stimulated steroidiogensis or estrogen activity of the compound. The body weights of the rats after 14 days treatment did not differ significantly from the control rats. The food consumption was reduced 6% in the treated rats. The LD_{50} of 2-(2,4-dimethylphenyl)indan-1,3-dione was greater than 500 mg/kg/day i.p. in mice as a single dose.

TABLE 6

Effects of 2-(2,4-Methylphenyl)indanone on Sprague-Dawley Rats ^{3}H-Cholesterol Distribution after 14 Days Administration at 20 Mg/Kg/Day Orally (N = 6)

	Organ wt(s)		DPM total organ	
Organ	Control	Treated	Control	Treated
Brain	0.500	0.543	23,400	27,400
Heart	0.447	0.410	49,400	46,800
Lung	0.477	0.457	84,100	72,900
Liver	4.167	3.807	329,800	285,300
Spleen	0.2567	0.243	26,300	8,700
Kidney	0.877	0.710	39,400	32,600
Stomach	0.757	0.550	40,700	19,700
Small intestine	3.587	2.953	76,100	127,300
Large intestine	0.890	1.223	108,310	131,700
Chyme	1.457	1.153	181,000	187,600
Feces	2.033	2.000	517,000	690,000
Reproductive tissue	1.767	1.5767	100,600	67,900
Adrenal	0.0501	0.0417	—	—
Plasma/ml	—	—	851	583
Urine/ml	—	—	1,960	1,770

*Standard deviations were within 5% of the tissues dpm values.

DISCUSSION

The 2-(2,4-dimethylphenyl)indan-1,3-dione derivative demonstrated hypolipidemic activity at the low dose of 20 mg/kg/day. The compound was more effective than clofibrate, which lowers serum cholesterol levels 15% and serum triglyceride levels 25% at 150 mg/kg/day in rats (25). The indan-1,3-dione derivative was equally effective pharmacologically by both oral and intraperitoneal administration, lowering both cholesterol and triglyceride

levels greater than 40%. In this respect, the agent demonstrated a profile that has been shown to be fairly standard for N-substituted cyclic imides in rodents.

The 2-(2,4-dimethylphenyl)indan-1,3-dione derivative suppressed the activity of enzyme involved in the early cytoplasmic synthesis of cholesterol and fatty acids, i.e., acetyl-CoA synthetase and ATP-dependent citrate lyase. Liver HMG-CoA reductase activity was not inhibited, which also has been observed for other cyclic imides (1-5). The 2-(2,4-dimethylphenyl)indan-1,3-dione derivative was a potent inhibitor of acyl-CoA acyl transferase activity at 20 mg/kg/day, which is the enzyme responsible for the synthesis and deposition of cholesteryl esters in tissues. Suppression of this enzyme would effectively reduce cholesterol storage in tissues. The rate-limiting enzymes of the de novo synthesis of triglycerides also were inhibited. In particular, phosphatidylate phosphohydrolase activity was suppressed by 2-(2,4-dimethylphenyl)indan-1,3-dione in a dose-dependent manner.

Lipids removed from the blood compartment in treated animals were not deposited in the organs of the body. The lipid reduction was demonstrated by the ^{3}H-cholesterol distribution study as well as the chemical analysis of lipids in the liver and small intestine where cholesterol and triglyceride levels were significantly reduced by drug treatment. The studies also suggested that the drug may cause excretion of cholesterol via the bile into the feces, thus clearing blood cholesterol from the body by this mechanism.

Serum lipoprotein lipid analysis showed that LDL-cholesterol levels were reduced significantly and HDL-cholesterol levels were significantly elevated after two wk of drug administration. Triglyceride and neutral lipids were reduced in HDL fractions. The modulation of the HDL/LDL cholesterol to this favorable ratio allows lipid clearance from peripheral tissue, e.g., aorta plaques and reduction of tissue cholesterol deposition from LDL. Nevertheless, other cyclic imides, e.g., phthalimide and saccharin, did not achieve this desired ratio within the two-wk administration of the drug. This ratio is better than that achieved by clofibrate, niacin, cholestyramine, oxandrolase or probucol in human treatment in which HDL cholesterol content was maximally raised only 4% to 16%. Supposedly, high cholesterol content of HDL and low cholesterol content of LDL protects man against myocardial infarction (6). Thus, the 2-(2,4-dimethylphenyl)indan-1,3-dione may have promise as a potential agent to use in atherosclerosis for therapeutic use, because the lipoprotein cholesterol content is modulated to a more favorable content for lipid clearance from the body.

ACKNOWLEDGMENT

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REFERENCES

1. Chapman, J.M. Jr., Cocolas, G.H., and Hall, I.H. (1979) *J. Med. Chem. 22,* 1399-1402.
2. Chapman, J.M. Jr., Cocolas, G.H., and Hall, I.H. (1983) *J. Med. Chem. 28,* 243-246.
3. Wyrick, S.D., Voorstad, P.J., Cocolas, G.H., and Hall, I.H. (1984) *J. Med. Chem. 27,* 768-772.
4. Murthy, A.R., Wyrick, S.D., Voorstad, P.J., and Hall, I.H. (1985) *Eur. J. Med. Chem. 20,* 547-550.
5. Murthy, A.R., Wyrick, S.D., and Hall, I.H. (1985) *J. Med. Chem. 28,* 1591-1596.
6. Mietinen, T.A., Huttunen, J.K., Strandberg, T., Nanlokarinen, V., Mattila, S., and Kumlin, T. (1981) *Lancet 2,* 478.
7. Ness, A.T., Pastewka, J.V., and Peacock, A.C. (1964) *Clin. Chem. Acta 10,* 229-237.
8. Goodridge, A.G. (1973) *J. Biol. Chem. 248,* 4328-4327.
9. Hoffman, M., Weiss, L., and Wieland, O.H. (1978) *Anal. Biochem. 84,* 441-448.
10. Robinson, B.H., Williams, G.R., Halperin, M.L., and Leznoff, C.C. (1971) *J. Biol. Chem. 246,* 5280-5286.
11. Robinson, B.H., Williams, G.R., Halperin, M.L., and Leznoff, C.C. (1970) *Eur. J. Biochem. 15,* 263-272.
12. Shefer, S., Hayser, S., and Mosbach, E.H. (1968) *J. Lipid Res. 9,* 328-333.
13. Balasubramanian, S., Mitropoulos, K.A., and Venkatesan, S. (1978) *Eur. J. Biochem. 90,* 377-383.
14. Haven, G.T., Krzenmien, J.R., and Nguyen, T.T. (1973) *Res. Commun. Chem. Path. Pharmacol. 6,* 253-261.
15. Wada, F., Hirate, K., and Sakamoto, Y. (1969) *J. Biochem. Tokyo 65,* 171-175.
16. Greenspan, M.D., and Lowenstein, J.M. (1968) *J. Biol. Chem. 243,* 6273-6280.
17. Lamb, R.G., Wyrick, S.D., and Piantadosi, C. (1977) *Atherosclerosis 27,* 147-154.
18. Mavis, R.D., Firkstein, J.N., and Hall, B.P. (1978) *J. Lipid Res. 19,* 467-477.
19. Chait, A., Iverius, P.H., and Brunzell, J.D. (1982) *J. Clin. Invest. 69,m* 490-493.
20. Folch, J., Lees, M., and Stanley, G.H.S. (1957) *J. Biol. Chem. 226,* 497-509.
21. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Phys. 37,* 911-917.
22. Bragdon, J.H. (1951) *J. Biol. Chem. 190,* 513-517.
23. Stewart, C.P., and Hendry, E.G. (1935) *Biochemistry 29,* 1683-1689.
24. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
25. Hall, I.H., Murthy, A.R.K., and Wyrick, S.D. (1986) *J. Pharm. Sci. 75,* 622-626.
26. Havel, R.J., Coler, H.A., and Bragdon, J.H. (1955) *J. Clin. Invest. 34,* 1345-1353.
27. Hatch, F.T., and Lees, R.S. (1968) *Adv. Lipid Res. 6,* 1-68.
28. Mookerjea, S., Parks, C.E., and Kuksis, A. (1975) *Lipids 10,* 374-382.

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Reaction of Cholesterol 5,6-Epoxides with Simulated Gastric Juice

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Cholesterol 5α,6α-epoxide (α-epoxide) and cholesterol 5β,6β-epoxide (β-epoxide) were individually suspended in simulated gastric juice (pH 1.2) at 37 C, and their reaction was followed by gradient high performance liquid chromatography (HPLC) with flame ionization (FID) detection. Both epoxides reacted rapidly in the aqueous acid medium. The α-epoxide formed 6β-chlorocholestane-3β,5α-diol (α-chlorohydrin) and 5α-cholestane-3β,5,6β-triol (triol), while the β-epoxide formed 5α-chlorocholestane-3β,6β-diol (β-chlorohydrin) and triol. The isomeric chlorohydrins reacted further to form the triol. In mildly alkaline aqueous medium, each chlorohydrin reverted to the epoxide from which it was formed. The data suggest that both epoxides, which have been reported to have adverse health effects in animals, would be largely hydrolyzed in the stomach and to the triol, which also has been reported to have biological activity. The data further suggest that residual chlorohydrins surviving stomach residence can be expected to revert to epoxide in the more alkaline intestinal environment.
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It is well-recognized that the exposure of cholesterol to oxidizing conditions gives rise to oxidation products (1), and many of these have been identified and now can be measured with some precision (2). A few of the major products of cholesterol oxidation have been reported to have adverse biological effects (3-12), and their implication in cardiovascular disease has been of concern. The possible adverse health effects of cholesterol oxidation products are highly relevant to food safety, because many cholesterol-containing foods are subjected to oxidizing conditions during various stages of processing, storage and/or preparation. Indeed, the problem may extend to foods that contain plant sterols, compounds that are closely related chemically to cholesterol and can be expected to give similar oxidation products, but this aspect has been studied only superficially.

The cholesterol oxidation products that have greatest relevance to food safety are the 5α,6α-epoxide, which has been reported to be carcinogenic and mutagenic (11) and the 3β,5α,6β-triol, which has been cited as being cytotoxic (6,9,10). The 5β,6β-epoxide also has recently been reported to be toxic (13), and because it is hydrated to form the triol at a faster rate than the 5α,6α-epoxide (14), its presence also must be considered pertinent to the food safety perspective.

The presence of cholesterol oxidation products in animal-derived foods has been reported with increasing frequency in recent years. Products that were reported to contain these cholesterol derivatives include heated tallow (15,16), dried egg preparations (17-21), butter and other dairy products (17,20,22-24), and other foods (17, 20,25,26). The documented existence of the 5,6-epoxides and of the triol in a large variety of foods raises the question of their fate upon ingestion.

Cholesterol 5α,6α-epoxide (α-epoxide) reacts with hydrochloric acid to give 6β-chlorocholestane-3β,5α-diol (α-chlorohydrin) (27,28). Similarly, cholesterol 5β,6β-epoxide (β-epoxide), on reaction with hydrochloric acid, yields 5α-chlorocholestane-3β,6β-diol (β-chlorohydrin) (28-31). Both chlorohydrins might be formed when mixtures of the isomeric cholesterol 5,6-epoxides are ingested. This paper reports a study of the reaction of the cholesterol epoxides with simulated gastric juice (pH 1.2) at 37 C.

EXPERIMENTAL

Materials and reagents. Cholesterol, 99+%, and α-epoxide were purchased from Sigma Chemical Co. (St. Louis, MO) and 5-cholestene-3β,25-diol was purchased from Research Plus, Inc. (Bayonne, NJ). β-Epoxide was prepared from cholesterol via 5α-cholestane-3β,5,6β-triol (triol) (32) and the corresponding triacetate (33) by the method of Chicoye et al. (34). Dulbecco's Modified Eagle Medium (high glucose) was purchased from Flow Laboratories (McLean, VA).

All solvents used were "distilled in glass grade" and for high performance liquid chromatography (HPLC) were degassed by vacuum filtration through a 0.2 μm filter. Water was double-deionized, glass distilled. Thin layer chromatography (TLC) plates, Silica Gel G and GHL (250 μ), were purchased from Analtech (Newark, DE).

Simulated gastric juice was prepared by diluting a solution of conc. HCl (0.82 ml, 12 M) and 0.411 g NaCl in distilled, deionized water to a total volume of 100 ml. The solution had a pH of 1.22. Phosphate buffer (pH 7.44) was a solution of monobasic potassium phosphate (KH_2PO_4, 0.5905 g) and dibasic sodium phosphate (Na_2HPO_4, 2.130 g) in 500 ml water.

PROCEDURES

Preparation of chlorohydrins: 6β-chlorocholestane-3β,5α-diol (3) (α-chlorohydrin). α-Epoxide (1) (501 mg) in a glass stoppered 500 ml Erlenmeyer flask was dissolved in ether (230 ml), and conc. HCl (125 ml) was added with cooling. The stoppered flask was allowed to stand at room temperature for 18 hr with magnetic stirring. After addition of 50 ml water and 50 ml ethyl acetate, the mixture was neutralized with 50% aqueous NaOH with cooling in ice to a pink phenolphthalein endpoint. The ether layer was separated, and the aqueous phase was extracted with 2 × 100 ml ethyl acetate. The combined organic phases were dried over anhydrous Na_2SO_4 and distilled on a rotary evaporator under aspirator vacuum to a dry residue (590 mg). The product was purified by semi-preparative HPLC using hexane/2-propanol (100:3, v/v).

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Abbreviations: α-Chlorohydrin, 6β-chlorocholestane-3β,5α-diol; α-epoxide, cholesterol 5α,6α-epoxide; β-chlorohydrin, 5α-chlorocholestane-3β,6β-diol; β-epoxide, cholesterol 5β,6β-epoxide; triol, 5α-cholestane-3β,5,6β-triol; FID, flame ionization detector; GC, gas chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

5α-Chlorocholestane-3β,6β-diol (4) (β-chlorohydrin). The method used to prepare the β-chlorohydrin from the β-epoxide (2) was identical to that used for the α-chlorohydrin.

Reaction of epoxides with simulated gastric juice: cholesterol 5α,6α-epoxide (1). To a 100 ml three-neck flask, equipped with magnetic stirring bar and a thermometer to which a thermoregulator was attached, 75.0 ml of simulated gastric juice was added. The flask was set on a stirring hot plate, and the temperature of the solution was regulated at 37.0 ± 0.5 C with vigorous agitation. α-Epoxide (21.03 mg) dissolved in 2-propanol (1000 μL) was injected slowly (3 min) below the surface of the stirred aqueous solution by means of a 500 μL syringe, resulting in a cloudy suspension. Aliquots (5 ml) were withdrawn from the reaction mixture immediately after the injection was completed and at periodic intervals thereafter by use of a volumetric pipette. Each aliquot was placed in a 20 ml screw-top vial containing 5 ml of ethyl acetate and 1 ml of a solution of internal standard (568 μg 5-cholesten-3β, 25 diol in 1 ml ethyl acetate). Vigorous shaking of the aliquot mixture was followed by phase separation and additional extractions (3 × 5 ml ethyl acetate) of the aqueous layer. Combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under a stream of N_2. The dry residue was dissolved in 30 μL 2-propanol, and the resulting solution was diluted with 1000 μL hexane in preparation for HPLC analysis.

Cholesterol 5β,6β-epoxide (2). The procedure used was the same as that used for the α-epoxide.

Reaction of chlorohydrins with simulated gastric juice: 6β-chlorocholestane-3β,5α-diol (3) (α) (α-chlorohydrin). The α-chlorohydrin was somewhat less soluble in 2-propanol than the α-epoxide, and 1500 μL 2-propanol were required to dissolve 24 mg of the α-chlorohydrin. Otherwise, the procedure described for the reaction of α-epoxide with simulated gastric juice was followed in detail.

5α-Chlorocholestane-3β,6β-diol (4) (β-chlorohydrin). The procedure used was identical to that described for the reaction of the α-epoxide, except that 24 mg of β-chlorohydrin was dissolved in 600 μL 2-propanol for injection in the reaction flask. The β-chlorohydrin appeared to be much less soluble in the aqueous phase than either of the epoxides or the α-chlorohydrin and formed large particles in the water layer and deposits on the outside of the injection needle.

Measurement of stability of the α-chlorohydrin (3) and the β-chlorohydrin (4) in Dulbecco's Modified Eagle Medium (high glucose), pH 7.35. The procedure described for the reaction of the α-epoxide with simulated gastric juice at 37 C was followed in detail, except that the aqueous medium was Dulbecco's Modified Eagle Medium (high glucose) rather than simulated gastric juice. Each chlorohydrin (~24 mg) was injected into the medium in a separate experiment, and product analysis was performed as described.

Measurement of the stability of α-chlorohydrin (3) in phosphate buffer, pH 7.44. α-Chlorohydrin (21.1 mg) in 2-propanol (1500 μL) was injected into 75 ml of phosphate buffer at 36 C, and product isolation and measurement were carried out as described above for the reaction of α-epoxide in simulated gastric juice.

Gas chromatography (GC). Analyses were performed as described (14).

Liquid chromatography (HPLC). The instrumentation used, including a flame ionization HPLC detector, has been described (35). Normal phase separations were performed on a 3.9 mm × 30 cm, 10 μm μ-Porasil column (Waters Associate, Framingham, MA) at ambient temperature. Separations of the four compounds of interest were achieved with a gradient solvent system at a total flow rate of 1.5 mL/min. The mobile phase consisted of solvent A, which was hexane/2-propanol (100:3, v/v), and solvent B, which was 2-propanol. At injection and for 10 min thereafter, the mobile phase was 100% A. The solvent composition then was changed linearly over a period of 10 min to 50% A and 50% B, stayed at that composition for five min and then changed linearly over a period of five min to 100% A to reach the starting point for the next analysis.

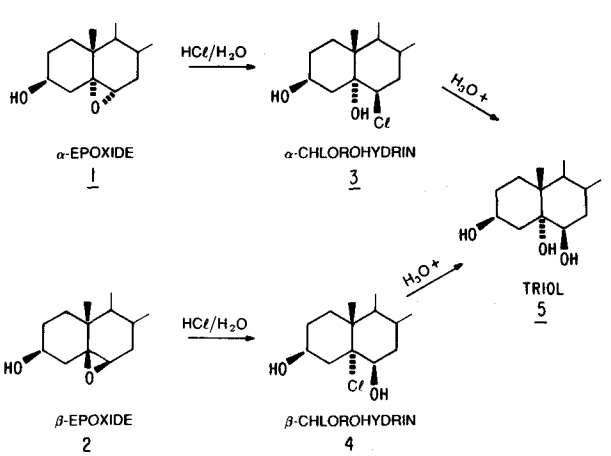
Thin layer chromatography. Before use, plates were washed by development with chloroform:methanol (2:1, v/v) and activated overnight in an air oven at 110 C. Spotted samples were developed with benzene:ethyl acetate:acetic acid (60:40:1, v/v/v). Air-dried plates were sprayed lightly with 50% sulfuric acid, placed on an unheated hot plate and gradually warmed to produce maximum color display of the cholesterol oxidation products, followed by complete charring at 220 C.

RESULTS AND DISCUSSION

Addition of hydrogen chloride to α-epoxide (1) and to β-epoxide (2) gave α-chlorohydrin (3) and β-chlorohydrin (4), respectively (Scheme 1).

Observation of the progress of the reaction in simulated gastric juice required analytical means to distinquish between the three expected components of these mixtures and to measure their concentrations. The components of interest were the epoxide and its corresponding chlorohydrin as well as triol (5).

The α-epoxide and its chlorohydrin were not resolvable by TLC with any of the several developing solvents tried. They gave single peaks by isocratic, normal phase HPLC and by capillary GC with or without prior silylation. Gradient, normal-phase HPLC gave excellent resolution of the three compounds of interest and the internal standard, 25-hydroxycholesterol (Fig. 1), with the use of a



SCHEME 1

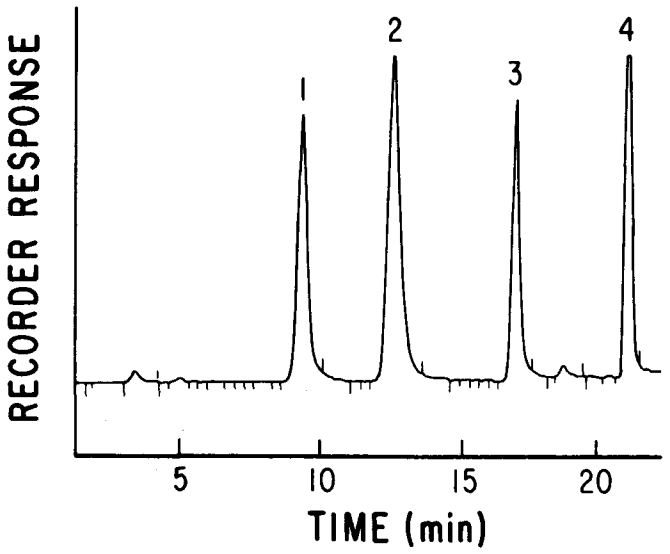


FIG. 1. Gradient HPLC of a four component mixture with FID detection (see Experimental). Peaks: (1) 5-cholestene-3β, 25 diol, (2) cholesterol 5α,6α-epoxide, (3) 6β-chlorocholestane-3β, 5α-diol, (4) 5α-cholestane-3β,5,6α-triol.

FID. The initial mobile phase was hexane/2-propanol (100:3, v/v) the polarity of which was increased linearly with 2-propanol. Response factors and linearity of response over a concentration range of 100 μg-0.39 μg per 100 μL were determined as reported (35) and permitted measurement of the relative concentrations of the components in the mixture from their area counts.

The β-epoxide and its chlorohydrin were readily resolved by TLC but not by GC. The gradient, normal-phase HPLC procedure developed for the α-epoxide and derived compounds was used without change to analyze mixtures of β-epoxide, β-chlorohydrin and triol, again with 25-hydroxycholesterol as internal standard.

Human gastric juice contains a variety of organic and inorganic constituents. The current study was confined to a simulated gastric juice consisting of an aqueous solution of hydrochloric acid and sodium chloride that approximates the concentration of these components in stimulated human gastric juice (36). A comparison of the cited composition and that of the present study is shown in Table 1.

Injection of the substrates (either of the two epoxides or either of the two chlorohydrins) into the vigorously stirred simulated gastric juice at 37 C was carried out slowly to minimize particle size of the resulting suspensions. Nevertheless, individual suspended particles were clearly visible and did not appear to change during the course of the reaction. Concentrations of the substrate in 75 ml reaction medium were chosen so that injection of 100 μL of an extract of each 5 ml aliquot produced a sizable signal on the HPLC-FID detector.

In early experiments, each 5 ml aliquot removed from the reaction mixture was immediately neutralized with concentrated sodium hydroxide to a phenolphthalein endpoint before the mixture was extracted with ethyl acetate. It was discovered, however, that this led to erroneous product compositions. Because the chlorohydrins are highly sensitive to base that converts them to epoxides, fleeting

TABLE 1

Composition of Gastric Juice

Component	Simulation of this study[a]	Simulated human gastric juice[b]
HCl	99.6 mM	0-135 mM
Cl^-	170 mM	131-170 mM
Na^+	70.3 mM	19-70 mM
pH	1.22	1.2-2.0

[a]Solution in double-deionized water.
[b]Ref. 36.

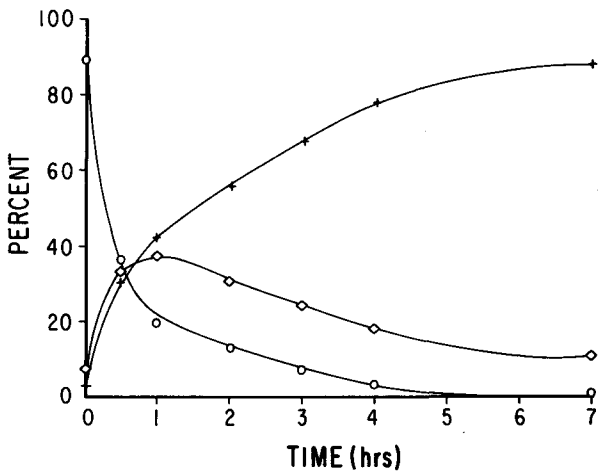


FIG. 2. Reaction of cholesterol 5α,6α-epoxide (α-epoxide) in simulated gastric juice at 37 C. (○) α-epoxide, (□) α-chlorohydrin, (+) triol.

local concentrations of base during the neutralization procedure gave unrealistically high concentrations of epoxides and low concentrations of chlorohydrins in these aliquots of the reaction mixture. The same sensitivity to base accounts for the presence of low concentrations of α-epoxide in the synthesized α-chlorohydrin where a neutralization step also is involved (see Experimental). The β-chlorohydrin is somewhat less sensitive to base-catalyzed dehydrohalogenation, so the synthesized β-chlorohydrin could be obtained free of contaminating β-epoxide. In the experiments reported here, neutralization with base before extraction was avoided (see Experimental).

The course of the reaction of the α-epoxide in simulated gastric juice is shown in Figure 2.

During the first two hr, the α-epoxide concentration decreased to less than 15%, and after four hr its concentration was less than 4%. Most foods are considered to have a residence time of two to four hr in the stomach (37). While the α-epoxide concentration decreased, the α-chlorohydrin concentration reached a maximum after about one hr and then decreased. Meanwhile the triol concentration increased steadily. The simultaneous decrease of the α-epoxide and the α-chlorohydrin accompanied by a steady increase in triol led to the expectation that a substantial portion of the α-chlorohydrin reacted further in the acid, aqueous medium to form triol. To test this

TABLE 2

Hydrolysis of α-Chlorohydrin and β-Chlorohydrin in Simulated Gastric Juice at 37 C

Time (hr)	α-Chlorohydrin			β-Chlorohydrin		
	Chlorohydrin (%)	Epoxide (%)	Triol[a] (%)	Chlorohydrin (%)	Epoxide (%)	Triol[a] (%)
0.0	93.4	3.0	3.6	99.2	—	0.8
0.5	69.3	4.3	26.4	96.7	—	3.3
1.0	51.6	3.3	45.1	92.8	—	7.2
2.0	30.6	2.2	67.2	89.0	—	11.0
3.0	18.2	1.3	80.5	85.4	—	14.6
4.0	16.0	0.9	83.1	84.3	—	15.7
7.0	6.9	0.5	92.6	78.6	—	21.4

[a]The same triol is formed from both chlorohydrins.

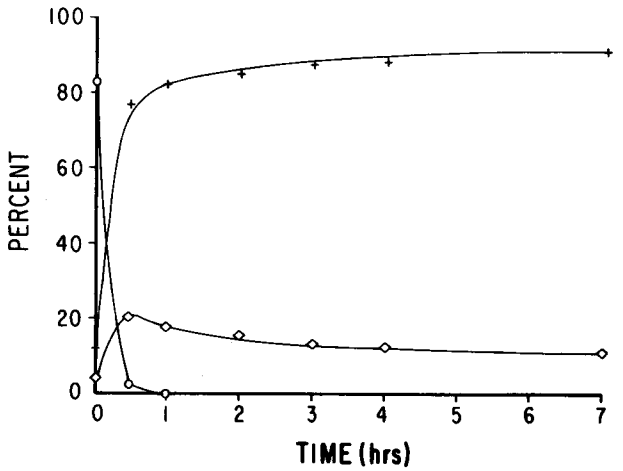


FIG. 3. Reaction of cholesterol 5β,6β-epoxide (β-epoxide) in simulated gastric juice 37 C. (○) β-epoxide, (□) β-chlorohydrin, (+) triol.

hypothesis, α-chlorohydrin was suspended in simulated gastric juice at 37 C. Results are shown in Table 2. It is apparent from these data that the α-chlorohydrin was indeed converted rather rapidly to triol. The small amount of α-epoxide that was present in the starting material as an impurity, was also mostly converted to triol. This latter conversion probably occurs via the α-chlorohydrin and directly, as well.

Exposure of the β-epoxide to the action of simulated gastric juice at 37 C caused a very rapid disappearance of epoxide from the mixture, as shown in Figure 3. The β-chlorohydrin peaked at only 1/2 hr into the experiment after it had reached the 20% level and then tapered off gradually, while the triol concentration rose steeply during the first hour. These data seem to indicate that much of the β-epoxide is converted directly to triol without the intermediacy of the β-chlorohydrin. The rapid hydration of the β-epoxide at pH 5.5 had been observed and measured earlier (14). Exposure of the β-chlorohydrin to simulated gastric juice at 37 C (Table 2) confirmed that the hydrolysis of the β-chlorohydrin was much slower than that of the α-chlorohydrin.

TABLE 3

Stability of Chlorohydrins in Growth Medium (pH 7.35)[a] at 37 C

Time (hr)	Epoxide (%)		Chlorohydrin (%)		Triol[b] (%)	
	α	β	α	β	α	β
0.0	4.6	0.7	93.6	98.9	1.8	0.4
0.5	28.5	5.5	69.4	93.4	2.1	1.1
1.0	32.4	7.3	65.7	91.2	1.9	1.5
2.0	33.6	10.1	64.5	87.5	1.9	2.4
7.0	38.2	16.9	59.3	78.4	2.5	4.7
24.0	46.8	32.1	50.3	59.5	2.9	8.4

[a]Dulbecco's Modified Eagle Medium (high glucose).
[b]The same triol is formed from both chlorohydrins.

TABLE 4

Stability of α-Chlorohydrin in Growth Medium (pH 7.35) and in Phosphate Buffer (pH 7.44), 37 C

Time (hr)	α-Epoxide (%)		α-Chlorohydrin (%)		Triol (%)	
	pH 7.35	pH 7.44	pH 7.35	pH 7.44	pH 7.35	pH 7.44
0.0	4.6	8.5	93.6	90.6	1.8	0.9
0.5	28.5	33.2	69.4	63.5	2.1	3.2
1.0	32.4	32.9	65.7	64.4	1.9	2.7
2.0	33.6	37.6	64.5	59.9	1.9	2.5
4.0	36.2	39.3	61.5	58.2	2.3	2.5
7.0	38.2	42.2	59.3	54.9	2.5	2.9
24.0	46.8	47.3	50.3	49.8	2.9	2.9

Stability of the isomeric chlorohydrins to mildly alkaline aqueous media is of concern, if their physiological activity is to be measured. Each of the chlorohydrins separately was placed into Dulbecco's Modified Eagle Medium (high glucose), which had a measured pH of 7.35. Results are shown in Table 3. It is clear that both chlorohydrins were converted to their respective epoxides at a very significant rate in this mildly alkaline medium. The conversion rate of the α-chlorohydrin appeared to be somewhat more rapid than that of the β-chlorohydrin, and surprisingly, a small amount of the latter was hydrolyzed to triol. Because this growth medium is a complex mixture of organic and inorganic ingredients, it was desirable to test the question whether dehydrohalogenation of the chlorohydrins was due to pH or possibly due to one or more of the components of the medium. Some of the α-chlorohydrin was suspended in phosphate buffer (pH 7.44) at 37 C, and the composition of the mixture was analyzed. The results are shown in Table 4 where the data at pH 7.35 (growth medium) and pH 7.44 (phosphate buffer) are compared. The similarity of the data in the two media confirms that the effect is due to pH rather than an ingredient of the growth medium. The comparison also serves as an indication of the reproducibility of the analytical procedure used in these experiments.

REFERENCES

1. Smith, L.L. (1981) *Cholesterol Autoxidation,* Plenum Press, New York.
2. Maerker, G. (1987) *J. Am. Oil Chem. Soc. 64,* 388-392.
3. Addis, P.B., Csallany, A.S., and Kindom, S.E. (1983) in *ACS Symposium Series No. 234* (Finley, J.W., and Schwass, D.E., eds.) pp. 85-98, American Chemical Society, Washington, D.C.
4. Imai, H., Werthessen, N.T., Subramanyam, V., LeQuesne, P., Soloway, A.H., and Kanisawa, M. (1980) *Science 207,* 651-653.
5. Kelsey, M.I., and Pienta, R.J. (1979) *Cancer Lett. 6,* 143-149.
6. Kelsey, M.I., and Pienta, R.J. (1981) *Toxicol. Lett. 9,* 177-182.
7. Hill, J.C., Peng, S.-K., Morin, R.J., and Taylor, C.B. (1984) *Exp. Mol. Pathol. 41,* 249-257.
8. Peng, S.-K., and Taylor, C.B. (1984) in *World Review of Nutrition and Dietetics* (Bourne, G.H., ed.) Vol. 44, pp. 117-154, S. Karger, Basel.
9. Peng, S.-K., Taylor, C.B., Hill, J.C., and Morin, R.J. (1985) *Atherosclerosis 54,* 121-133.
10. Peng, S.-K., Morin, R.J., Tham, P., and Taylor, C.B. (1985) *Artery 13,* 144-164.
11. Sevanian, A., and Peterson, A.R. (1984) *Proc. Natl. Acad. Sci. USA 81,* 4198-4202.
12. Smith, L.L., Smart, V.B., and Made Gowda, N.M. (1986) *Mutat. Res. 161,* 39-48.
13. Raaphorst, G.P., Azzam, E.I., Langlois, R., and VanLier, J.E. (1987) *Biochem. Pharmacol. 36,* 2369-2372.
14. Maerker, G., and Bunick, F.J. (1986) *J. Am. Oil Chem. Soc. 63,* 771-777.
15. Park, S.W., and Addis, P.B. (1986) *J. Agric. Food Chem. 34,* 653-659.
16. Bascoul, J., Domerque, N., Ole, M., and Crastes de Paulet, A. (1986) *Lipids 21,* 383-387.
17. Finocchiaro, E.T., and Richardson, T. (1983) *J. Food Prot. 46,* 917-925.
18. Tsai, L.-S., and Hudson, C.A. (1985) *J. Food Sci. 50,* 229-231, 237.
19. Missler, S.R., Wasilchuk, B.A., and Merritt, C. Jr. (1985) *J. Food Sci. 50,* 595-598, 646.
20. Fischer, K.-H., Laskawy, G., and Grosch, W. (1985) *Z. Lebensm. Unters. Forsch. 181,* 14-19.
21. Sugino, K., Terao, J., Murakami, H., and Matsushita, S. (1986) *J. Agric. Food Chem. 34,* 36-39.
22. Csiky, I. (1982) *J. Chromatog. 241,* 381-389.
23. Luby, J.M., Gray, J.I., Harte, B.R., and Ryan, T.C. (1986) *J. Food Sci. 51,* 904-907, 923.
24. Finocchiaro, E.T., Lee, K., and Richardson, T. (1984) *J. Am. Oil Chem. Soc. 61,* 877-883.
25. Lee, K., Herian, A.M., and Higley, N.A. (1985) *J. Food Prot. 48,* 158-161.
26. Higley, N.A., Taylor, S.L., Herian, A.M., and Lee, K. (1986) *Meat Sci. 16,* 175-188.
27. Barton, D.H.R., and Miller, E. (1950) *J. Am. Chem. Soc. 72,* 370-374.
28. Rivett, D.E.A., and Wallis, E.S. (1950) *J. Org. Chem. 15,* 35-41.
29. Windaus, A. (1921) Hoppe-Seyler's *Z. Physiol. Chem. 117,* 146-155.
30. Baxter, R.A., and Spring, F.S. (1943) *J. Chem. Soc.,* 613-615.
31. Plattner, P.A., and Lang, W. (1944) *Helv. Chim. Acta 27,* 1872-1875.
32. Fieser, L.F., and Rajagopalan, S. (1949) *J. Am. Chem. Soc. 71,* 3938-3941.
33. Davis, M., and Petrow, V. (1949) *J. Chem. Soc.,* 2536-2539.
34. Chicoye, E., Pourie, W.D., and Fennema, O. (1968) *Lipids 3,* 335-339.
35. Maerker, G., Nungesser, E.H., and Zulak, I.M. (1988) *J. Agric. Food Chem. 15,* 61-63.
36. Leach, A.A. (1961) in *Biochemists' Handbook* (Long, C., ed.) pp. 911-914, D. Van Nostrand Company, Inc., Princeton, NJ.
37. Hawk, P.B., Oser, B.L., and Summerson, W.H. (1954) in *Practical Physiological Chemistry,* 13th edn., p. 368, McGraw Hill Book Co., New York.

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Dietary Manipulation of Macrophage Phospholipid Classes: Selective Increase of Dihomogammalinolenic Acid

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Because alterations in the dietary content of fatty acids are an important method for modulating macrophage eicosanoid production, we have quantitated the levels of n-6 and n-3 polyunsaturated fatty acids in peritoneal macrophage individual phospholipids from mice fed diets (3 wk) with either safflower oil (SAF), predominantly containing 18:2n-6, borage (BOR) containing 18:2n-6 and 18:3n-6, fish (MFO) containing 20:5n-3 and 22:6n-3, and borage/fish mixture (MIX) containing 18:2n-6, 18:3n-6, 20:5n-3 and 22:6n-3. Dietary n-3 fatty acids were readily incorporated into macrophage phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). The increase in n-3 fatty acid levels was accompanied by a decrease in the absolute levels of 18:2n-6, 20:4n-6 and 22:4n-6 in PC, PE and PS. Interestingly, PI 20:4n-6 levels were not significantly lowered ($P > 0.05$) in MIX and MFO macrophages relative to SAF and BOR. These data demonstrate the unique ability of this phospholipid to selectively maintain its 20:4n-6 levels. In BOR and MIX animals, 20:3n-6 levels were significantly increased ($P < 0.05$) in all phospholipids relative to SAF and MFO. The combination of borage and fish oils (MIX diet) produced the highest 20:3n-6/20:4n-6 ratio in all phospholipids. These data show that the macrophage eicosanoid precursor levels of 20:3n-6, 20:4n-6 and n-3 acids can be selectively manipulated through the use of specific dietary regimens. This is noteworthy because an increase in phospholipid levels of 20:3n-6 and 20:5n-3, while concomitantly reducing 20:4n-6, may have therapeutic potential in treating inflammatory disorders. *Lipids 23,* **766–770 (1988).**

Macrophages are a highly diverse population of cells capable of executing or modifying a number of important biological functions. Because of their abundance, distribution and versatility, macrophages can influence almost every aspect of the immune and inflammatory responses (1). Because macrophages contain high levels of arachidonic acid (20:4n-6) in their phospholipids, it is likely that macrophage-derived eicosanoids are involved in inflammation and cellular regulation (2). In addition, the eicosanoids also may play an important role in the autoregulation of several macrophage functions (3–6).

It is well-established that alterations in the dietary content of fatty acids are an important method for modulating eicosanoid production because, in general, eicosanoid biosynthesis is dependent upon the size of the fatty acid precursor pool(s) (7,8). In earlier studies (9), we have demonstrated that fish oil feeding qualitatively increases murine peritoneal macrophage total phospholipid levels of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), while borage oil, containing linoleic acid (18:2n-6) and γ-linolenic acid (18:3n-6), increases the macrophage dihomo-γ-linolenic acid (20:3n-6) to 20:4n-6 ratio. This is noteworthy because the manipulation of eicosanoid precursor levels of 20:3n-6, 20:4n-6 and 20:5n-3 could potentially attenuate the phlogistic response (10–12).

Previous studies have proposed different classes of phospholipids as sources of eicosanoid fatty acid precursors (13,14). Therefore, the distribution of n-3 and n-6 fatty acids among individual phospholipids in macrophages may be important in determining the quantitative release of these potential eicosanoid antecedents (14). As yet, no attempt has been made to quantitate macrophage individual phospholipid fatty acid levels following borage or borage/fish oil feeding. The purpose of the present work was to quantitate the levels of n-6 and n-3 polyunsaturated fatty acids in peritoneal macrophage individual phospholipids from mice fed either safflower oil (SAF) predominantly containing 18:3n-6, borage oil (BOR) containing 18:2n-6 and 18:3n-6, menhaden fish oil (MFO) containing 20:5n-3 and 22:6n-3, and borage/fish oil mixture (MIX) containing 18:2n-6, 18:3n-6, 20:5n-3 and 22:6n-3.

MATERIALS AND METHODS

Materials. Fatty acid methyl ester standards were from NuChek Prep. (Elysian, MN). Diheptadecanoyl-L-alpha-phosphatidylcholine was from Sigma Chemical Co. (St. Louis, MO). Silica gel 60 plates were from E. Merck (Darmstadt, FRG). The dietary oils were from: safflower, California Oils (Richmond, CA); borage, Traco Labs (Champaign, IL); and menhaden fish oil, Zapata-Haynie (Reedville, VA). ACS-grade organic solvents were from Fisher Scientific (Chicago, IL).

Animals and dietary treatments. Pathogen-free female C57BL/6NCR mice (Charles River, Wilmington, MA), weighing 15–20 g were used. All mice had been fed a commercial pelleted mouse diet (Ralston-Purina, St. Louis, MO) and were housed (6–8 animals per cage) in autoclaved cages with laminar flow hoods to minimize spontaneous macrophage activation (15). On day zero of the experiment, the mice were randomly assigned to one of four purified diets for three wk. The diet varied only in the type of oil fed, i.e., either safflower, borage, fish or borage/fish mixture in a 2.5/7.5 ratio at 10% of the diet by weight. All dietary components were purchased from ICN Nutritional Biochemicals (Cleveland, OH), except where noted. The basic compositions of the purified diet by weight was casein, 20%; DL-methionine, 0.3%; corn starch, 15%; sucrose, 44%; cellulose, 5%; mineral mix, 3.5% (16); vitamin mix, 2.0% (16); choline chloride, 0.2%; and the appropriate dietary oil, 10%. The fatty acid composition of the different dietary oils are shown in Table 1. The diets were adequate in all nutrients, except MFO, which was deficient in 18:2n-6 (17). A diurnal light cycle of 12 hr was maintained, and food and autoclaved water were available ad libitum. The powered diets were mixed and stored under nitrogen at −20 C. Fresh diet was provided daily to each group to minimize auto-oxidation. Diets were

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Abbreviations: BOR, borage; FAME, fatty acid methyl esters; MFO, fish; MIX, borage/fish mixture; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SAF, safflower oil; TLC, thin layer chromatography.

TABLE 1

Fatty Acid Composition of Dietary Oils

Fatty acid	SAF	BOR	MIX	MFO
14:0	tr	tr	6.3	8.7
16:0	6.9	9.3	15.0	16.7
16:1n-7	tr	tr	8.3	11.2
18:0	2.2	3.0	2.7	2.8
18:1n-9	11.1	13.5	9.0	7.0
18:2n-6	75.9	39.7	10.9	1.2
18:3n-6	tr	26.4	7.0	0.6
20:4n-6	tr	tr	0.7	0.7
20:5n-3	tr	tr	11.8	15.2
22:5n-3	tr	tr	1.6	2.0
22:6n-3	tr	tr	6.2	7.8

Results are expressed as mg/100 mg total fatty acids present. Only the major fatty acids are presented. tr, Trace amounts, less than 0.1%.

periodically checked by determination of the fatty acid composition. The profiles did not vary significantly in the diets over the three-wk feeding period. Body weights were recorded weekly, and the food intakes of the diets were diurnally monitored and not found to be significantly ($P > 0.05$) different (data not shown).

Macrophage cultures. Mice were injected intraperitoneally with 2 ml of thioglycollate broth (Difco, Detroit, MI) prepared to manufacturer's specification, three days before killing. Primary cultures of peritoneal macrophages were established from responsive cells as described (18).

Lipid analysis. Macrophage monolayers were scraped from the culture dish in 3 ml of chilled methanol and extracted by the method of Folch (19). All solvents contained 0.01% butylated hydroxytoluene to prevent oxidative breakdown. The individual phospholipids were separated by thin layer chromatography (TLC) on silica gel 60 plates using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, v/v/v/v) (20). The purified phospholipids so obtained represented 90.3 ± 1.4% (mean ± SEM, n = 13) of the phospholipid present in the original total lipid extract as judged by recovery of radioactivity from endogenously labeled phospholipid from mouse peritoneal macrophages. Bands were detected under ultraviolet light after spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol. A known concentration of diheptadecanoyl-L-alpha-phosphatidylcholine as an internal standard was added to the isolated phospholipids before transmethylation (21,22). The resultant fatty acid methyl esters (FAME) were further purified on silica gel 60 plates run in a benzene solvent system and detected by dichlorofluorescein. The corresponding FAME bands were scraped and extracted using 2 ml methanol, 2 ml petroleum ether and 1 ml water. The concentration and fatty acid composition of the isolated phospholipids were determined after gas chromatographic analysis of the derived FAME as described (21,22). Replicate cell monolayers were solubilized in 0.1 M sodium hydroxide for protein determination using a modified Lowry method (23) with bovine serum albumin as the standard. Data were subjected to one-way analysis of variance using multiple comparisons, with the upper level of significance chosen at $P < 0.05$.

RESULTS

The data in Table 1 show that the four diets differed dramatically in the relative percentage of n-6 fatty acids (18:2n-6 and 18:3n-6) and n-3 fatty acids (20:5n-3 and 22:6n-3). BOR contained 26% by weight 18:3n-6, while this fatty acid represented less than 1% of the total acids present in either SAF or MFO diets. MFO was characterized by its content of 20:5n-3 (15%) and 22:6n-3 (8%). As expected, the MIX diet contained a blend of 18:2n-6 (11%), 18:3n-6 (7%), 20:5n-3 (12%) and 22:6n-3 (6.2%) as the major polyunsaturated fatty acids. No differences ($P > 0.05$) between the diets were noted with respect to the numbers or relative proportions of resultant peritoneal exudate populations after thioglycollate injection (data not shown). The effects of SAF, BOR, MIX and MFO supplementation on macrophage individual phospholipid fatty acid composition are shown in Tables 2–5. BOR and MIX feeding consistently increased 20:3n-6 levels ($P < 0.05$) relative to SAF and MFO in phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). PC and PE contained the highest absolute levels of 20:3n-6. Although the macrophage PI 20:3n-6 concentration was significantly increased after feeding 18:3n-6 rich diets, the relative increment was smallest among the phospholipids examined (Table 5). Interestingly, 18:3n-6 was not detected in PC, PE, PS and PI from mice fed BOR and MIX. These results are consistent with our earlier findings (9), in which 18:3n-6 was not detected in macrophage total phospholipids in mice fed BOR. Levels of 20:5n-3, 22:5n-3, and 22:6n-3 were significantly higher ($P < 0.05$) in PC, PE, PS and PI in MFO and MIX macrophages. The increase in the n-3 fatty acid levels was accompanied by a decrease in the absolute levels of 18:2n-6, 20:4n-6 and 22:4n-6 in PC, PE and PS. In contrast, PI 20:4n-6 was not significantly lowered ($P > 0.05$) in MIX and MFO diets relative to SAF and BOR. The present study also demonstrates that 22:4n-6 is a major polyenoic acid in SAF and BOR PC, PE and PS. Only trace amounts of 22:4n-6 were observed in PI.

Figure 1 shows the 20:3n-6/20:4n-6 ratios in macrophage individual phospholipids. BOR and MIX diets,

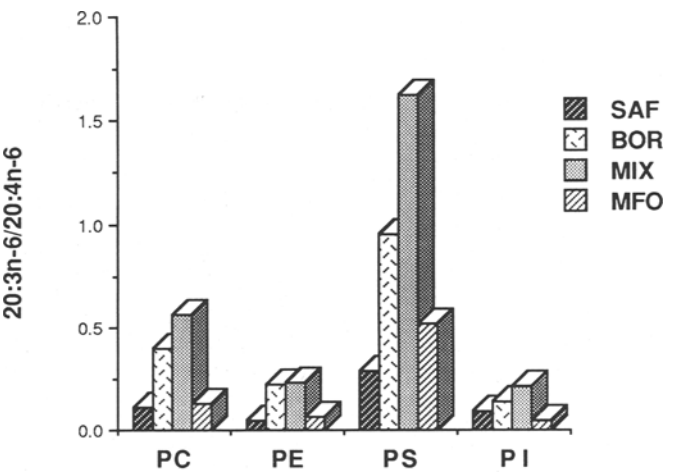

FIG. 1. 20:3n-6/20:4n-6 ratios in macrophage individual phospholipids. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI).

TABLE 2

Fatty Acids in Macrophage Phosphatidylcholine

Fatty acid	SAF	BOR	MIX	MFO
16:0	9.55 ± 0.37[a]	10.98 ± 0.54[a]	10.78 ± 0.58[a]	11.96 ± 0.68[a]
16:1n-7	0.26 ± 0.06[a]	0.31 ± 0.06[a]	0.66 ± 0.09[b]	0.96 ± 0.16[b]
18:0	2.76 ± 0.18[a,b]	3.22 ± 0.10[c]	2.56 ± 0.08[a]	2.45 ± 0.00[a]
18:1n-9	2.42 ± 0.18[a]	2.72 ± 0.01[a]	2.80 ± 0.25[a]	4.22 ± 0.18[b]
18:1n-7	0.71 ± 0.01[a]	0.76 ± 0.07[a]	1.12 ± 0.11[b]	1.27 ± 0.16[b]
18:2n-6	5.16 ± 0.28[c]	2.87 ± 0.01[a]	2.08 ± 0.27[a]	1.12 ± 0.02[b]
18:3n-6	tr[a]	tr[a]	tr[a]	tr[a]
20:2n-6	0.91 ± 0.03[b]	tr[a]	tr[a]	tr[a]
20:3n-6	0.42 ± 0.01[a]	1.94 ± 0.26[c]	1.14 ± 0.00[b]	0.24 ± 0.04[a]
20:4n-6	3.72 ± 0.22[b]	4.90 ± 0.04[c]	2.05 ± 0.12[a]	1.88 ± 0.14[a]
20:5n-3	tr[a]	tr[a]	0.98 ± 0.14[b]	1.82 ± 0.06[c]
22:4n-6	1.22 ± 0.02[b]	1.37 ± 0.10[b]	tr[a]	tr[a]
22:5n-3	tr[a]	tr[a]	1.59 ± 0.03[b]	2.23 ± 0.04[c]
22:6n-3	tr[a]	tr[a]	0.06 ± 0.03[b]	1.01 ± 0.08[b]

Results are expressed as μg fatty acid/mg protein. Each figure represents the mean ± SEM from six to eight animals. Values with the same superscripts are not significantly different ($P > 0.05$). tr, Trace amounts, less than 0.1 μg fatty acid/mg protein.

TABLE 3

Fatty Acids in Macrophage Phosphatidylethanolamine

Fatty acid	SAF	BOR	MIX	MFO
16:0	1.12 ± 0.02[a]	1.14 ± 0.01[a]	1.28 ± 0.07[a]	1.01 ± 0.38[a]
16:1n-7	tr[a]	tr[a]	tr[a]	tr[a]
18:0	3.20 ± 0.12[a]	3.49 ± 0.12[a]	3.16 ± 0.21[a]	3.54 ± 0.14[a]
18:1n-9	0.42 ± 0.12[a]	0.56 ± 0.01[a]	0.62 ± 0.02[a]	0.69 ± 0.16[a]
18:1n-7	0.18 ± 0.04[a]	0.24 ± 0.12[a,b]	0.24 ± 0.00[a,b]	0.30 ± 0.01[b]
18:2n-6	0.96 ± 0.02[c]	0.56 ± 0.02[a]	0.45 ± 0.03[a]	0.22 ± 0.06[b]
18:3n-6	tr[a]	tr[a]	tr[a]	tr[a]
20:2n-6	tr[a]	tr[a]	tr[a]	tr[a]
20:3n-6	0.16 ± 0.06[a]	0.89 ± 0.01[c]	0.54 ± 0.06[b]	0.11 ± 0.04[a]
20:4n-6	3.64 ± 0.02[c]	4.02 ± 0.06[c]	2.34 ± 0.06[b]	1.78 ± 0.26[a]
20:5n-3	tr[a]	tr[a]	0.98 ± 0.02[b]	1.74 ± 0.36[c]
22:4n-6	2.00 ± 0.06[b]	2.37 ± 0.11[b]	0.12 ± 0.12[a]	tr[a]
22:5n-3	tr[a]	tr[a]	2.04 ± 0.42[b]	2.04 ± 0.14[b]
22:6n-3	tr[a]	tr[a]	1.26 ± 0.04[b]	1.17 ± 0.12[b]

Refer to Table 2 for legend details. Results are expressed as μg fatty acid/mg protein.

TABLE 4

Fatty Acids in Macrophage Phosphatidylserine

Fatty acid	SAF	BOR	MIX	MFO
16:0	0.57 ± 0.03[a]	0.58 ± 0.06[a]	0.59 ± 0.01[a,b]	0.71 ± 0.03[b]
16:1n-7	tr[a]	tr[a]	0.05 ± 0.03[a]	0.04 ± 0.01[a]
18:0	2.91 ± 0.03[a]	3.22 ± 0.17[a]	2.87 ± 0.34[a]	2.81 ± 0.31[a]
18:1n-9	0.33 ± 0.03[a]	0.37 ± 0.03[a]	0.45 ± 0.03[b]	0.65 ± 0.01[c]
18:1n-7	0.08 ± 0.02[a]	0.08 ± 0.00[a]	0.12 ± 0.00[a,b]	0.16 ± 0.01[b]
18:2n-6	0.76 ± 0.10[c]	0.42 ± 0.04[b]	0.30 ± 0.01[a,b]	0.16 ± 0.01[a]
18:3n-6	tr[a]	tr[a]	tr[a]	tr[a]
20:2n-6	0.12 ± 0.02[b]	tr[a]	tr[a]	tr[a]
20:3n-6	0.15 ± 0.03[a]	0.56 ± 0.08[b]	0.42 ± 0.01[b]	0.10 ± 0.04[a]
20:4n-6	0.52 ± 0.01[b]	0.59 ± 0.04[b]	0.26 ± 0.02[a]	0.22 ± 0.02[a]
20:5n-3	tr[a]	tr[a]	0.08 ± 0.04[b]	0.18 ± 0.01[c]
22:4n-6	0.70 ± 0.10[b]	0.70 ± 0.10[b]	tr[a]	tr[a]
22:5n-3	tr[a]	tr[a]	0.62 ± 0.02[b]	0.82 ± 0.14[b]
22:6n-3	tr[a]	tr[a]	0.32 ± 0.01[b]	0.33 ± 0.01[b]

Refer to Table 2 for legend details. Results are expressed as μg fatty acid/mg protein.

TABLE 5

Fatty Acids in Macrophage Phosphatidylinositol

Fatty acid	SAF	BOR	MIX	MFO
16:0	0.23 ± 0.01[a]	0.28 ± 0.00[a]	0.28 ± 0.04[a]	0.38 ± 0.01[b]
16:1n-7	tr[a]	tr[a]	tr[a]	tr[a]
18:0	1.68 ± 0.06[b]	1.70 ± 0.01[b]	1.28 ± 0.03[a]	1.75 ± 0.10[b]
18:1n-9	0.10 ± 0.02[a]	0.14 ± 0.02[a,b]	0.14 ± 0.02[a,b]	0.18 ± 0.01[b]
18:1n-7	0.02 ± 0.01[a]	0.02 ± 0.01[a]	0.04 ± 0.01[a]	0.04 ± 0.01[a]
18:2n-6	0.14 ± 0.02[b]	0.10 ± 0.01[a,b]	0.10 ± 0.02[a,b]	0.03 ± 0.01[a]
18:3n-6	tr[a]	tr[a]	tr[a]	tr[a]
20:2n-6	tr[a]	tr[a]	tr[a]	tr[a]
20:3n-6	0.10 ± 0.02[a]	0.15 ± 0.01[b]	0.20 ± 0.02[b]	0.04 ± 0.00[a]
20:4n-6	1.11 ± 0.04[a]	1.10 ± 0.04[a]	1.00 ± 0.12[a]	0.88 ± 0.10[a]
20:5n-3	tr[a]	tr[a]	0.15 ± 0.03[b]	0.16 ± 0.02[b]
22:4n-6	0.08 ± 0.08[a]	tr[a]	tr[a]	tr[a]
22:5n-3	tr[a]	tr[a]	0.28 ± 0.01[c]	0.22 ± 0.03[b]
22:6n-3	tr[a]	tr[a]	0.09 ± 0.01[b]	0.16 ± 0.02[c]

Refer to Table 2 for legend details. Results are expressed as μg fatty acid/mg protein.

each containing 18:3n-6, significantly increased ($P < 0.05$) PC, PE, PS and PI 20:3n-6/20:4n-6 ratios relative to SAF and MFO. Interestingly, the combination of BOR and MFO oils, i.e. MIX diet, produced the highest ratios of 20:3n-6/20:4n-6. The MIX ratios in decreasing order were PS, 1.62 ± 0.12; PC, 0.56 ± 0.04; PE, 0.23 ± 0.02; and PI, 0.21 ± 0.05. Although MIX PC, PE and PS 20:3n-6 levels were lower than BOR, 20:4n-6 levels were competitively reduced through substitution by n-3 fatty acids.

DISCUSSION

We have demonstrated that the total phospholipid fatty acid profile of mouse peritoneal macrophages can be dramatically manipulated by BOR and MFO feeding (9). The data suggested that 20:3n-6 and 20:5n-3, normally found in much smaller amounts than 20:4n-6 in cellular phospholipids and therefore not considered to be major precursors for eicosanoid formation in the macrophage, could following dietary manipulation serve as important substrates for the cyclooxygenase and lipoxygenase enzymes (10,11,24). However, to date, detailed studies documenting the ability of BOR or borage/fish (MIX) dietary manipulation to change macrophage individual phospholipid fatty acid concentrations (μg/mg protein) are lacking. The present study includes such an examination, focusing on the nebulous relationship of BOR and MIX feeding on quantitative fatty acid changes in mouse peritoneal macrophages.

The data presented demonstrate that dietary n-3 fatty acids were readily incorporated into macrophage PC, PE, PS and PI. The increases in 20:5n-3 and 22:6n-3 were associated with a concomitant reduction in PC, PE and PS 20:4n-6 levels. These results are consistent with previous macrophage n-3 feeding studies (25,26). Interestingly, PI 20:4n-6 levels were not significantly lowered in MIX and MFO macrophages relative to SAF and BOR. Unfortunately, there are no analogous quantitative data on macrophage PI with which to compare the present results. The inability of BOR, MIX or MFO to influence 20:4n-6 levels in PI demonstrate the unique ability of this phospholipid to selectively maintain its polyunsaturated fatty acid composition. The retention of 20:4n-6 in macrophage PI is of interest because it is a major eicosanoid precursor (27). In addition, the accumulation of n-3 fatty acids in PC, PE, PS and PI is noteworthy because n-3 fatty acid-enriched macrophages secrete reduced amounts of 20:4n-6-derived eicosanoids (25,26).

Comparison of the fatty acid composition of macrophage phospholipids in mice fed 18:3n-6 rich diets (BOR, MIX), showed that 20:3n-6 levels were significantly increased in PC, PE, PS and PI, relative to SAF and MFO. The fatty acid profile changes following BOR and MIX feeding, specifically the absence of 18:3n-6 and the elevated 20:3n-6/20:4n-6 ratios, support our earlier in vitro observations in which the presence of an active long chain elongase and modest Δ5 desaturase activities in the peritoneal macrophage were documented (18,24). The selective accumulation of 20:3n-6 is of particular interest because this fatty acid can serve as a precursor for the biosynthesis of the 1-series prostaglandins (8) that possess antiphlogistic properties (11,28).

The combination of 18:2n-6, 18:3n-6, 20:5n-3 and 22:6n-3 (MIX diet), produced the highest 20:3n-6/20:4n-6 ratio in all phospholipids, relative to SAF, MFO and BOR diets. These data are consistent with earlier studies (29,30), in which n-3 fatty acids were shown to inhibit the Δ5 desaturation of 20:3n-6 to 20:4n-6. In addition, the reduction in the absolute levels of 18:2n-6, 20:4n-6, and 22:4n-6 in MIX and MFO PC, PE and PS, also demonstrates the ability of n-3 fatty acids to competitively reduce n-6 fatty acid levels through substitution. Although only a single MIX treatment of borage and fish oil was examined, the results suggest that the 20:3n-6/20:4n-6 levels can be significantly manipulated through combined oil feeding. Therefore, it is possible to markedly increase the macrophage phospholipid levels of 20:3n-6, an eicosanoid precursor with antiphlogistic potential, while concomitantly reducing 20:4n-6, whose oxidative metabolism via the cyclooxygenase and lipoxygenase pathways plays a role in inflammation.

In summary, data from this study demonstrate that the peritoneal macrophage eicosanoid precursor levels of

20:3n-6, 20:4n-6, and n-3 acids can be selectively manipulated through the use of specific dietary regimens. This possibility underscores the need for further experimentation in elucidating the potential of these alterations to attenuate the phlogistic response.

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REFERENCES

1. Somers, S.D., Johnson, W.J., and Adams, D.O. (1986) in *Cancer Immunology: Innovative Appraoches to Therapy* (Herberman, R., ed.) pp. 69-122, Martinus Nijhoff, Boston, MA.
2. Stenson, W.F., and Parker, C.W. (1980) *J. Immunol. 125,* 1-6.
3. Schultz, R.M., Parlidis, N.A., Stylos, W.A., and Chirigos, M.A. (1979) *Science 202,* 320-321.
4. Snyder, D.S., Beller, D.I., and Unanue, E. (1982) *Nature London 299,* 163-165.
5. Taffet, S.M., and Russell, S.W. (1981) *J. Immunol. 126,* 424-427.
6. Schnyder, J., Dewald, B., and Baggiolini, M. (1981) *Prostaglandins 22,* 411-421.
7. Mathias, M.M., and Dupont, J. (1985) *Lipids 20,* 791-801.
8. Willis, A.L. (1981) *Nutr. Rev. 39,* 289-301.
9. Chapkin, R.S., Somers, S.D., Schumacher, L., and Erickson, K.L. (1988) *Lipids 23,* 380-383.
10. Terano, T., Salmon, J.A., Higgs, G.A., and Moncada, S. (1986) *Biochem. Pharmacol. 35,* 779-785.
11. Kunkel, S.L., Ogawa, H., Ward, P.A., and Zurier, R.B. (1981) *Prog. Lipid Res. 20,* 885-889.
12. Horrobin, D.F. (1983) *Rev. Pure Appl. Sci. 4,* 339-383.
13. Brown, M.L., Jakubowski, J.A., Leventis, L.L., and Deykin, D. (1987) *Biochim. Biophys. Acta 921,* 159-166.
14. Mahadevappa, V.G., and Holub, B.J. (1987) *J. Lipid Res. 28,* 1275-1280.
15. Meltzer, M.S. (1976) *Cell Immunol. 75,* 176-186.
16. Erickson, K.L., Schlanger, D.S., Adams, D.A., Fregeau, D.R., and Stern, J.S. (1984) *J. Nutr. 114,* 1834-1842.
17. National Research Council (1978) in *Nutrient Requirements of Laboratory Animals,* Vol. 10, pp. 38-53, National Academy of Sciences, Washington, D.C.
18. Chapkin, R.S., Somers, S.D., and Erickson, K.L. (1988) *J. Immunol. 140,* 2350-2355.
19. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 22,* 497-509.
20. Holub, B.J., and Skeaff, C.M. (1987) *Meth. Enzymol. 141,* 234-244.
21. Chapkin, R.S., Haberstroh, B., Liu, T., and Holub, B.J. (1983) *J. Lab. Clin. Med. 101,* 726-735.
22. Chapkin, R.S., Ziboh, V.A., Marcelo, C.L., and Voorhees, J.J. (1986) *J. Lipid Res. 27,* 945-954.
23. Schmidt, G., Martin, A.P., Stuhlman, R.A., Townsend, J.F., Lucas, F.V., and Vorbeck, M.L. (1984) *Lab. Invest. 30,* 451-457.
24. Chapkin, R.S., Miller, C.C., Somers, S.D., and Erickson, K.L. (1988) *Biochim. Biophys. Acta 959,* 322-331.
25. Lokesh, B.R., Hsieh, H.L., and Kinsella, J.E. (1986) *J. Nutr. 116,* 2547-2552.
26. Magrum, L.J., and Johnston, P.V. (1983) *Lipids 18,* 514-521.
27. Chensue, S.W., and Kunkel, S.L. (1983) *Clinics Lab. Med. 3,* 677-694.
28. Kernoff, P.B.A., Willis, A.L., Stone, K.J., Davies, J.A., and McNicol, G.P. (1977) *Br. Med. J. 2,* 1441-1444.
29. Takahashi, R., Nassar, B.A., Huang, Y.S., Begin, M.E., and Horrobin, D.F. (1987) *Throm. Res. 47,* 135-146.
30. Juan, H., and Sametz, W. (1985) *Naunyn-Schmied. Arch. Pharmacol. 329,* 388-393.

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Lipid Abnormalities in Pancreatic Tissue of Streptozotocin-induced Diabetic Rats

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Pancreatic lipid and fatty acid composition was determined in streptozotocin-induced diabetic rats and compared to control and insulin-treated diabetic rats. A pronounced decrease of total fatty acids was recorded and mainly accounted for by a drop of fatty acids in glycerides. Cholesterol, on the other hand, was significantly increased two- to three-fold, leading to an elevated cholesterol/phospholipid ratio. Morpho-cytochemical studies confirmed these findings because the multiple lipid droplets present in acinar cells of diabetic animals were found to be of cholesterol nature. The major alterations in phospholipid-fatty acid composition were characterized by an increase of linoleate coupled to a decrease of monounsaturates and arachidonate, suggesting defective metabolism of saturates and of linoleate. This was further supported by fatty acid ratios that suggested low Δ5 and Δ9 desaturation. Daily administration of insulin for 10 days restored and overcorrected the various lipid alterations. This study suggests that there are alterations in lipid composition and metabolism in the exocrine pancreas of chronic streptozotocin-induced diabetic rats. Their possible role in the mechanism regulating pancreatic function and secretion remains to be elucidated. ***Lipids 23,*** **771-778 (1988).**

Modifications in the secretory activity of the exocrine pancreas have been reported in diabetic humans (1-3), as well as in experimental diabetic animals (4-9). In addition, frequent lesions such as generalized fatty degeneration of the acini are pancreatic histologic changes characteristic of both latent and overt diabetes (10-12). Earlier studies suggested that these alterations, and the impairment of amylase secretion in particular, result from the disturbance of the high insulin supply to the acinar cells by the islet-acini portal system (9,13-15).

Diabetes is commonly associated with increased levels of all plasma lipids (16,17). This hyperlipidaemia reflects more of an increase of the saturated and monounsaturated than of the polyunsaturated fatty acids (18). Recently, attention has been drawn to similar lipid abnormalities in different tissues such as heart, liver, testes and intestine of diabetic animals (19-21). The decrease in the relative proportion of the polyunsaturated fatty acids, mainly arachidonic acid, was determined in these tissues and was found to result from an impairment of desaturation activity. The changes in lipid composition might affect the morphological and functional integrity of the pancreas in diabetes (20,22), an observation that has been established for testes and seminal vesicles (20).

The present studies were designed to investigate the effect of streptozotocin-induced diabetes on the lipid and fatty acid profile of the pancreas of diabetic rats. By means of fatty acid ratios, the capacity of the tissue to elongate and desaturate fatty acids was evaluated. Furthermore, pancreatic tissue was studied by a morphological-cytochemical approach. Finally, the ability of insulin to normalize the biochemical and morphological changes was examined.

MATERIALS AND METHODS

Animals. The experiments were performed on male Sprague-Dawley rats weighing 100 g at the beginning of the experiment. Diabetes in 12 rats was induced by i.v. injection of 50 mg/kg body wt of streptozotocin (Boehringer Mannheim, Quebec, Canada) freshly dissolved in 10 mmol/l sodium citrate (pH 4.5). Six control rats were given the vehicle alone. The animals had been maintained on a standard laboratory chow and tap water ad libitum for three months. A group of six streptozotocin-injected animals demonstrating both hyperglycemia and glucosuria were treated with a daily injection of insulin (NPH, Connaught Laboratories, Ontario, Canada; 8 U/day per animal) for 10 days. With this treatment, normal homeostasis was reestablished. These animals received their 10th injection two hr before killing. At the time of decapitation, the different groups of rats were fasted overnight. Blood was collected on ethylenediamine tetraacetic acid (EDTA) and separated into plasma for various biochemical determinations.

Tissue lipid extraction. Immediately after the rats had been exsanguinated, the pancreas were excised. The tissues were minced, extensively washed in 0.15 mol/l cold NaCl solution, blotted, weighed and extracted in chloroform/methanol (2:1, v/v) (23). To ensure that total lipids were extracted, the samples were subjected to an additional extraction with chloroform/methanol.

Thin layer chromatographic separation and gas liquid chromatography analysis. The chloroform phase was evaporated under a gentle stream of nitrogen at 24 C. The lipid components were either directly methylated for analysis of fatty acid distribution in total lipids or first separated on thin layer chromatography plates (Silica Gel G) with a solvent system containing hexane/diethyl ether/glacial acetic acid (80:20:3, v/v/v). The different spots were identified with the help of standards that comigrated on the plate. The cholesteryl ester (CE), triglyceride (TG), free fatty acid (FFA), diglyceride (DG), monoglyceride (MG) and phospholipid (PL) fractions were scraped into small glass tubes and transesterified as described (24). Supernatant aliquots, including internal standard, were injected into a 60 m fused silica column with an internal diameter of 0.32 mm and coated with

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Abbreviations: CE, cholesterol ester; DG, diglyceride; EDTA, ethylenediamine tetraacetic acid; FA, fatty acid; FC, free cholesterol; HDL, high density lipoprotein; MG, monoglyceride; LDL, low density lipoprotein; PL, phospholipid; TC, total cholesterol; TG, triglyceride.

0.20 mm of SP-2331. Analyses were performed on a Hewlett-Packard No. 5880 gas chromatograph equipped with a flame ionization detector. Standard mixtures of fatty acids (Sigma Chemical Co., St. Louis, MO) were used to determine the retention times and the calculation of correction factors. The area under the chromatogram peaks was calculated with the aid of integrator, calibrated with the standard methylated fatty acid mixtures.

Calculation of desaturation activities. The activity of desaturase enzymes was not measured directly but inferred from calculation of immediate product to substrate ratios of fatty acids. The following fatty acid ratios were selected because in all chromatograms they were present and reliably quantitated; the index of Δ5 desaturase activity was 20:4ω6/20:3ω6, and that of Δ9 was 18:1ω9/18:0. In the calculation of Δ4 and Δ6 desaturase activities, one elongase for each was included. We were forced to use 22:6ω3/20:5ω3 and 20:3ω6/18:2ω6 for the Δ4 and for the Δ6 desaturates because the substrates 22:5ω3 and 18:3ω6 were not always present on each chromatogram.

Other analytical methods. Plasma triglycerides, total cholesterol and free cholesterol determinations were carried out by enzymatic kits from Boehringer (Mannheim, Quebec, Canada). Glucose levels were determined by the glucose oxidase method and insulin by radioimmunoassay.

Morphological studies. For studies at the electron microscope level, small fragments of pancreatic tissue were sampled from the dorsal and ventral parts of the pancreas of the different groups of animals and fixed by immersion in 0.1 M phosphate-buffered 1% glutaraldehyde for two hr at room temperature. The tissue fragments then were post-fixed with 1% osmium tetroxide and embedded in Epon 812 according to standard procedures. Thin sections were cut, stained with uranyl acetate and lead citrate and examined with the electron microscope. The cytochemical staining of cholesterol was performed using modifications of the technique proposed by Frühling et al. (25). In short, after fixation with glutaraldehyde and osmium tetroxide, the tissue fragments were dehydrated in ethanol 30% and 70% (15 and 30 min, respectively), infiltrated in ethanol 70%/Epon (l/l) twice 30 min and finally embedded in Epon and polymerized at 60 C. The thin sections were lightly stained with uranyl acetate and examined. For the light microscopy immunocytochemical investigation of endocrine cells in the islets of Langerhans, the pancreatic tissues were fixed with Bouin's fluid and embedded in paraffin according to standard techniques. Sections (5 μm thick) were stained, applying the indirect immunofluorescence technique, for the demonstration of insulin, glucagon and somatostatin containing cells as described (13). Specific antibodies directed towards these pancreatic hormones were used (13).

Statistical analysis. Analysis of variance and the multiple comparison procedure of Scheffe (26) were used to test for differences between means. If F was significant at the 0.05 level, the difference between means was evaluated by the method of least significant differences.

RESULTS

At the time of killing, three months after the streptozotocin injection, the diabetic rats had significantly higher plasma glucose as well as TG and TC concentrations than control and insulin-treated rats (Table 1). On the other hand, their insulin levels were significantly depressed to about 32% of normal values, while those of insulin-treated diabetic animals showed a marked increase above control values.

The composition of plasma fatty acids is reported in Table 2. The proportion of linoleic acid (18:2ω6) was higher, while that of α-linolenic acid (18:3ω3) was lower in diabetic rats. No significant difference could be demonstrated in the monounsaturates and the arachidonate (20:4ω6), although a decrease was noted for both. Table 2 also demonstrates that streptozotocin diabetes produced changes in fatty acid levels consistent with a 55% inhibition of Δ5 desaturation. However, the 18:1ω9/18:2ω6 ratio, an index of essential fatty acid deficiency, was found to be similar to that of controls, while the 18:2ω6/20:4ω6 ratio indicative of fatty acid elongation and both Δ5 and Δ6 desaturation was higher, although not significantly. In contrast, the percentages of comparable fatty acids were corrected in diabetic rats treated with insulin, except for 18:3ω3. Insulin therapy altered the relative content of some fatty acids such as 15:0, 16:1ω7 and 20:3ω9 and corrected the Δ4 and Δ5 desaturations indices to normal values.

Lipid fatty acid concentration in pancreatic tissue. Table 3 presents the concentrations of fatty acids in the various lipid classes found in the pancreas. There was a significant decrease in the total fatty acid content in the diabetic state. This change was largely accounted for by a marked decrease of TG (~35-fold). The only significant ($P < 0.05$) increment in pancreatic fat content was in the

TABLE 1

Biochemical Plasma Parameters in Control, Diabetic and Insulin-treated Hyperglycemic Rats

Rats	Glucose (mg/dl)	TG (mg/dl)	TC (mg/dl)	Insulin (U/ml)
Controls	159 ± 9	65 ± 9	72 ± 6	36.6 ± 2.2
Diabetics	535 ± 42[a]	384 ± 70[a]	112 ± 9[a]	24.8 ± 3.0[a]
Insulin-treated diabetics	37 ± 2[b,c]	42 ± 12[c]	38 ± 7[b,c]	81.3 ± 1.7[b,c]

Values represent means ± SEM of plasma concentrations obtained from 10-12 donor rats.

[a]Significant difference between the control and diabetic rats.

[b]Significant difference between the control and insulin-treated diabetics.

[c]Significant difference between the diabetics and insulin-treated diabetics.

TABLE 2

Total Plasma Fatty Acids

Fatty acid	Controls	Diabetics	Insulin-treated diabetics
		Wt. %	
12:0	0.14 ± 0.01	0.12 ± 0.07	0.16 ± 0.02
14:0	0.56 ± 0.03	0.53 ± 0.13	0.62 ± 0.05
15:0	0.39 ± 0.04	0.61 ± 0.09	0.28 ± 0.02[c]
16:0	24.29 ± 0.98	24.08 ± 0.85	27.08 ± 1.45
16:1ω7	0.77 ± 0.03	0.44 ± 0.12	2.41 ± 0.22[b,c]
17:0	0.40 ± 0.01	0.69 ± 0.06[a]	0.28 ± 0.007[c]
18:0	12.66 ± 0.40	12.14 ± 0.80	13.01 ± 0.67
18:1ω9	12.17 ± 0.65	10.95 ± 0.89	12.30 ± 1.17
18:1ω7	2.27 ± 0.05	1.77 ± 0.12	2.37 ± 0.29
18:2ω6	22.26 ± 0.53	26.66 ± 1.32[a]	17.92 ± 0.37[b,c]
18:3ω3	0.57 ± 0.03	0.33 ± 0.03[a]	0.38 ± 0.03[b]
18:3ω6	0.20 ± 0.03	0.28 ± 0.04	0.11 ± 0.01[c]
20:2ω6	0.25 ± 0.02	0.28 ± 0.05	0.21 ± 0.02
20:3ω6	0.57 ± 0.07	0.85 ± 0.09	0.74 ± 0.09
20:3ω9	0.11 ± 0.02	0.04 ± 0.01	0.27 ± 0.08[c]
20:4ω6	17.54 ± 1.46	12.49 ± 2.02	14.58 ± 2.30
20:5ω3	0.91 ± 0.11	1.02 ± 0.15	1.60 ± 0.46
22:3ω3	0	0.16 ± 0.05	0
22:4ω6	0.23 ± 0.04	0.21 ± 0.02	0.18 ± 0.04
22:5ω3	0.75 ± 0.13	0.86 ± 0.05	0.73 ± 0.15
22:5ω6	0	0.42 ± 0.09	0.62 ± 0.05
22:6ω3	3.08 ± 0.36	5.32 ± 0.77	4.21 ± 0.72
18:1ω9/18:2ω6	0.55 ± 0.03	0.41 ± 0.02	0.69 ± 0.08[c]
18:2ω6/20:4ω6	1.30 ± 0.14	2.34 ± 0.45	1.35 ± 0.25
Δ4 desaturation[d]	3.41 ± 0.22	6.20 ± 2.21	3.02 ± 0.58
Δ5 desaturation[e]	32.30 ± 4.72	14.68 ± 1.74[a]	20.58 ± 4.60
Δ6 desaturation[f]	0.026 ± 0.003	0.032 ± 0.004	0.041 ± 0.005
Δ9 desaturation[g]	0.97 ± 0.15	0.93 ± 0.14	0.95 ± 0.08

Values are means ± SEM for four animals in each group, expressed as percentage of total plasma fatty acids.

[a]Significant difference between the control and diabetic tissue.

[b]Significant difference between control and diabetic insulin-treated.

[c]Significant difference between diabetic and diabetic insulin-treated.

[d]Index of Δ4 desaturation activity: 22:6ω3/20:5ω3.

[e]Index of Δ5 desaturation activity: 20:4ω6/20:3ω6.

[f]Index of Δ6 desaturation activity: 20:3ω6/18:2ω6.

[g]Index of Δ9 desaturation activity: 18:1ω9/18:0.

TABLE 3

Fatty Acid Concentration of Lipid Components in Pancreatic Tissue

Rats	Total fatty acids	TG	DG	MG	FFA	CE	PL
				μg/g tissue			
Controls	10249.3 ± 2879.7	9668.0 ± 4998.1	65.2 ± 20.4	216.4 ± 133.9	68.8 ± 1.7	26.6 ± 10.0	754.5 ± 192.6
Diabetics	2190.9 ± 264.1[a]	271.2 ± 111.8	37.3 ± 9.5	187.8 ± 99.8	41.9 ± 15.0	92.3 ± 41.3[a]	1111.9 ± 157.9
Insulin-treated + diabetics	7513.3 ± 1207.1	5344.1 ± 1413.7	81.8 ± 16.8	141.1 ± 41.7	60 ± 12	37.3 ± 4.3[b]	985.5 ± 99.1

Values are means ± SEM for four animals in each group, expressed in μg/g tissue. TG, triglyceride; DG, diglyceride; MG, monoglyceride; FFA, free fatty acid; CE, cholesteryl ester; PL, phospholipid.

[a]Significant difference between the control and diabetic tissue.

[b]Significant difference between the diabetic and diabetic insulin treated.

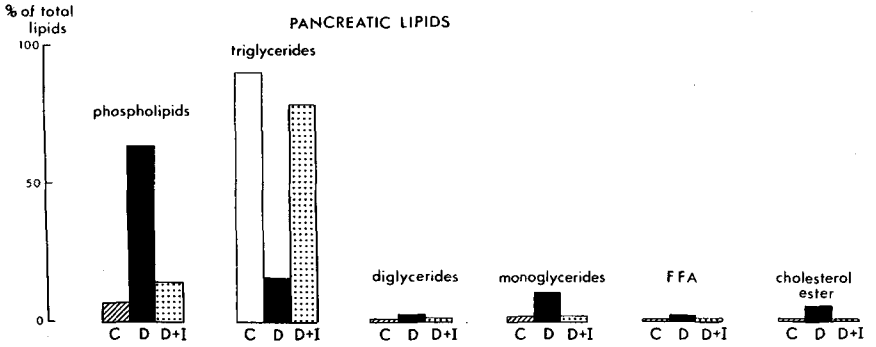


FIG. 1. Percent distribution of lipid classes in pancreatic tissue. The different lipid classes were extracted by chloroform/methanol (2:1, v/v), separated by thin layer chromatography, transesterified, and injected to GLC as described in Materials and Methods.

CE fraction (~3-fold increase). Treatment of rats with insulin led to a restoration of normal lipid concentrations. This suggests that the changes noted in the diabetic condition are the result of insulin deficiency.

Distribution of lipid components in pancreatic tissue. When values of the various lipid components were expressed as percentages of total amount of fat recovered from the pancreatic tissue, an abnormal pattern was obtained in the diabetic state (Fig. 1). As expected from Table 3, a substantial decrease of TG is noted and is associated with a relative increase of the other lipid classes, the phospholipid fraction being predominantly affected. On the other hand, insulin-treated diabetic animals demonstrated the same profile as controls.

Nonesterified cholesterol concentration in pancreatic tissue. Besides the cholesterol increment recorded in Table 3, a significant increase of nonesterified cholesterol was found in pancreatic tissues of diabetic animals (Fig. 2). When the ratio of cholesterol/phospholipid was calculated, it was found to be significantly higher in diabetic (5-fold) than in control rats. Again, treatment with insulin abolished the large difference between the normal and the diabetic state.

Fatty acid distribution in total lipids of pancreatic tissue. The fatty acid profile of the total lipids extracted from the pancreas is recorded in Table 4. The pattern observed in diabetics differs markedly from that of controls. The major essential fatty acids (18:2ω6, 20:4ω6) together with other polyunsaturates (20:3ω6, 20:5ω3) were higher, while most saturates (except 18:0) and monounsaturates were lower. The decreased oleic acid could be the result of the diminished Δ9 desaturation, while Δ5 desaturation was found to be significantly decreased as inferred from fatty acid changes in the diabetic animals. Insulin administration partly corrected these abnormalities.

Fatty acid profile of phospholipids. Fatty acid analysis of PL of pancreatic tissue was performed. Table 5 shows that linoleic acid and 20:3ω6 were increased and arachidonic acid was decreased, indicating an impaired utilization of linoleic and 20:3ω6 acid in diabetic animals. This led to a three-fold increase of the 18:2ω6/20:4ω6 ratio and a decrease of the ω3 fatty acid family, which together could be secondary to the apparent decrease of Δ5 desaturation. In addition to the diminished proportions of 14:0, 15:0 and 16:0, altered percentages of monounsaturates

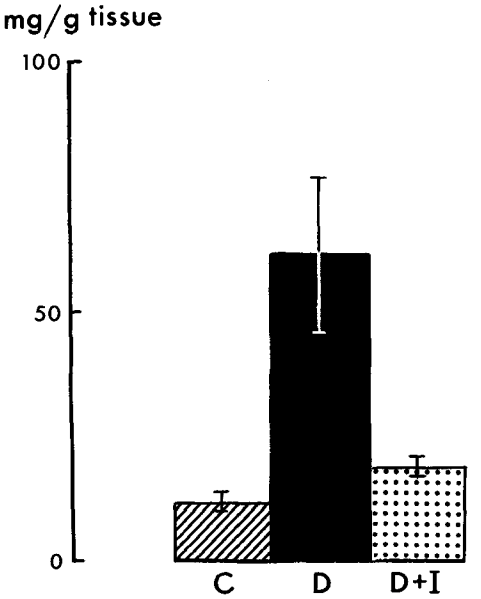


FIG. 2. Free cholesterol concentration in pancreatic tissue.

(16:1ω7 and 18:1ω9) were noticed, which is the likely result of a diminished Δ9-desaturation responsible for the conversion of saturated fatty acids to monounsaturates. Treatment of the diabetic animals with insulin corrected or even overcorrected the abnormal fatty acid pattern of the PL fraction without changing the decreased Δ5 fatty acid desaturation.

Morphological studies. Immunofluorescence studies performed on the endocrine portion of the pancreas demonstrated a drastic decrease in the number of insulin-containing cells in the islets of Langerhans of the streptozotocin-induced diabetic animals when compared with the controls (results not illustrated). By electron microscopy, the acinar cells in control animals appeared pyramidal in shape, with the nucleus in basal position surrounded by a well-developed rough endoplasmic reticulum arranged in parallel rows (Fig. 3). The Golgi apparatus and numerous secretory zymogen granules were present in a supra-nuclear position, close to the apical plasma membrane. Lipid droplets, in particular, were small in size and very few in number. When present, they were located

TABLE 4

Pancreatic Fatty Acid Composition

Fatty acids	Controls	Diabetics	Insulin-treated diabetics
		Wt. %	
14:0	1.58 ± 0.20	0.58 ± 0.04[a]	1.48 ± 0.07[c]
15:0	0.37 ± 0.02	0.36 ± 0.02	0.23 ± 0.04[b,c]
16:0	24.37 ± 0.66	23.64 ± 0.57	30.84 ± 0.64[b,c]
16:1ω7	2.23 ± 0.40	0.41 ± 0.04	8.34 ± 0.89[b,c]
18:0	7.99 ± 0.76	20.06 ± 1.18[a]	6.79 ± 1.03[c]
18:1ω9	27.83 ± 1.80	11.98 ± 1.04[a]	29.20 ± 3.18[c]
18:2ω6	28.94 ± 0.79	31.80 ± 0.54[a]	16.57 ± 0.63[b,c]
18:3ω3	1.53 ± 0.22	0.24 ± 0.05[a]	0.77 ± 0.07[b]
20:3ω6	0.32 ± 0.04	1.24 ± 0.07[a]	0.62 ± 0.27[c]
20:4ω6	4.39 ± 0.95	9.46 ± 0.92[a]	4.42 ± 1.55[c]
20:5ω3	0.61 ± 0.12	0.99 ± 0.21	0.78 ± 0.29
Saturated	34.30 ± 1.27	44.54 ± 1.02[a]	39.31 ± 1.55[b,c]
Polyunsaturated	35.64 ± 0.79	43.31 ± 1.08[a]	23.15 ± 2.57[b,c]
ω3	2.04 ± 0.23	1.14 ± 0.28	1.55 ± 0.23
ω6	33.60 ± 0.77	42.50 ± 1.01[a]	21.60 ± 2.35[b,c]
ω9	27.83 ± 1.80	11.98 ± 1.04[a]	29.20 ± 3.18[c]
ω6/ω9	1.23 ± 0.09	3.77 ± 0.44[a]	0.86 ± 0.23[c]
18:1ω9/18:2ω6	0.96 ± 0.06	0.38 ± 0.04[a]	1.80 ± 0.24[b,c]
18:2ω6/20:4ω6	9.96 ± 3.46	3.58 ± 0.37	5.99 ± 1.36
Δ5 desaturation[d]	17.29 ± 3.99	7.74 ± 0.79[a]	7.83 ± 1.47[b]
Δ6 desaturation[e]	0.011 ± 0.001	0.039 ± 0.002	0.035 ± 0.013
Δ9 desaturation[f]	3.73 ± 0.55	0.61 ± 0.07[a]	5.05 ± 1.00[b]

Values are means ± SEM for four animals in each group, expressed as percentage of fatty acids recovered from pancreatic tissue.

[a]Significant difference between the control and diabetic tissue.

[b]Significant difference between control and diabetic insulin-treated.

[c]Significant difference between diabetic and diabetic insulin-treated.

[d]Index of Δ5 desaturation activity: 20:4ω6/20:3ω6.

[e]Index of Δ6 desaturation activity: 20:3ω6/18:2ω6.

[f]Index of Δ9 desaturation activity: 18:1ω9/18:0.

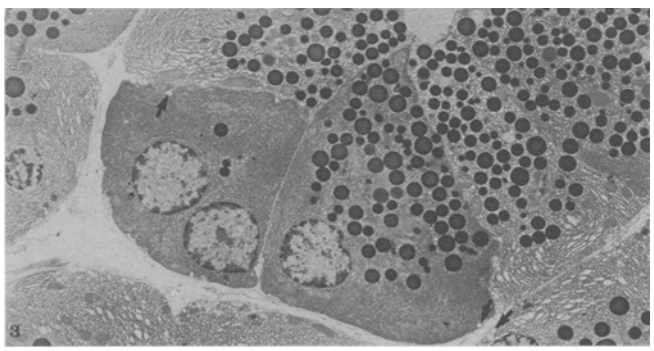


FIG. 3. Pancreatic tissue from a control animal. The acinar cells display their typical morphology with numerous secretory zymogen granules in the supranuclear portion of the cell. Lipid droplets (arrows) are few in number and small in size. ×2000.

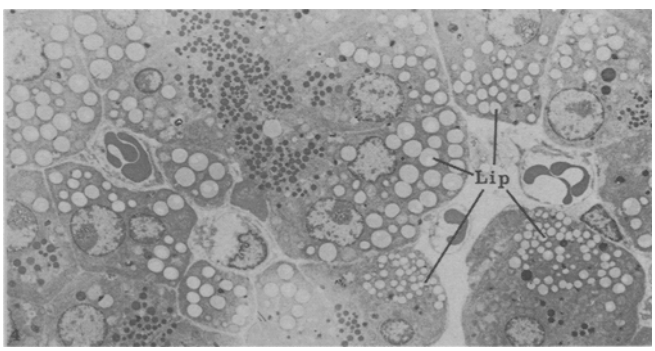


FIG. 4. Pancreatic tissue from a diabetic animal. The acinar cells display accumulations of lipid droplets (Lip) some of which are large in size. ×1100.

at the basal part of the cell (Fig. 3). In contrast, the acinar cells of the diabetic animals appeared infiltrated with large amounts of lipid droplets (Fig. 4). These were quite variable in size, some of them reaching 2000 nm in diameter. Because of these large aggregates of lipid droplets, the morphology of the cells was somewhat altered, in particular the arrangement of the rough endoplasmic reticulum at the basal part of the cell (Fig. 4). The Golgi apparatus and the secretory granules remained located towards the apical region of the cell. The

TABLE 5

Pancreatic Phospholipid Fatty Acids

	Controls	Diabetics	Insulin-treated diabetics
		Wt. %	
14:0	0.28 ± 0.01	0[a]	0.34 ± 0.05[c]
15:0	0.27 ± 0.03	0[a]	0.21 ± 0.01[c]
16:0	31.41 ± 1.46	25.76 ± 0.74[a]	32.52 ± 0.59[c]
16:1ω7	0.32 ± 0.08	0[a]	0.84 ± 0.07[b,c]
18:0	18.35 ± 0.28	19.84 ± 1.04	15.18 ± 0.38[b,c]
18:1ω9	7.52 ± 0.19	7.04 ± 0.21	6.75 ± 0.29
18:2ω6	19.23 ± 0.27	34.65 ± 0.36[a]	22.10 ± 0.76[b,c]
18:3ω3	0	0	0
20:3ω6	0.75 ± 0.15	1.38 ± 0.04	2.24 ± 0.26[b,c]
20:4ω6	20.99 ± 0.73	10.94 ± 0.79[a]	17.08 ± 1.21[c]
20:5ω3	1.69 ± 0.15	0.52 ± 0.08[a]	2.73 ± 0.20[b,c]
Saturated	50.17 ± 1.60	45.60 ± 0.50[a]	48.25 ± 0.58
Polyunsaturated	42.07 ± 1.37	47.37 ± 0.64[a]	44.15 ± 0.91
ω3	1.72 ± 0.18	0.52 ± 0.08[a]	2.73 ± 0.20[b,c]
ω6	40.78 ± 1.02	46.97 ± 0.64[a]	41.43 ± 0.78[c]
ω9	7.52 ± 0.19	7.04 ± 0.21	6.75 ± 0.29
ω6/ω9	5.42 ± 0.11	6.70 ± 0.27	6.21 ± 0.35
18:1ω9/18:2ω6	0.391 ± 0.005	0.203 ± 0.006[a]	0.307 ± 0.013[b,c]
18:2ω6/20:4ω6	0.92 ± 0.03	3.23 ± 0.27[a]	1.34 ± 0.15[c]
Δ5 desaturation[d]	30.74 ± 6.38	7.89 ± 0.49[a]	8.57 ± 1.85[b]
Δ6 desaturation[e]	0.039 ± 0.007	0.040 ± 0.001	0.102 ± 0.012[b,c]
Δ9 desaturation[f]	0.41 ± 0.01	0.36 ± 0.01[a]	0.45 ± 0.01[c]

Values are means ± SEM for four animals in each group, expressed as percentage of fatty acids recovered from pancreatic tissue.

[a]Significant difference between the control and diabetic tissue.

[b]Significant difference between control and diabetic insulin-treated.

[c]Significant difference between diabetic and diabetic insulin-treated.

[d]Index of Δ5 desaturation activity: 20:4ω6/20:3ω6.

[e]Index of Δ6 desaturation activity: 20:3ω6/18:2ω6.

[f]Index of Δ9 desaturation activity: 18:1ω9/18:0.

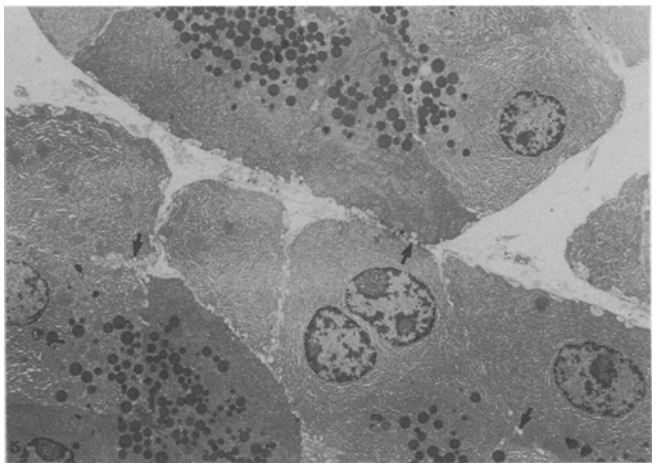


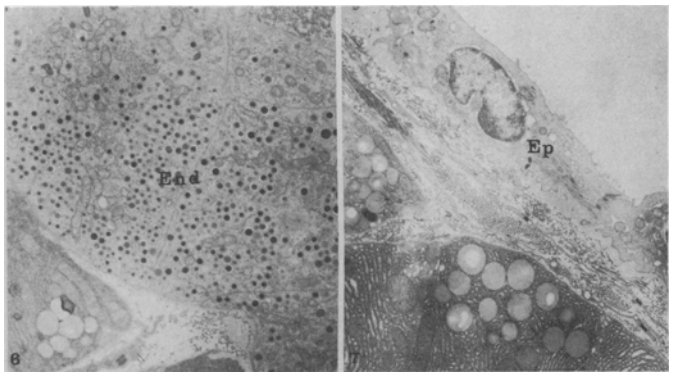
FIG. 5. Pancreatic tissue from an insulin-treated diabetic animal. Small lipid droplets (arrows) are present in the acinar cells. ×1500.

accumulation of lipid droplets in the acinar cells was found in both the ventral and dorsal parts of the pancreas. The acinar cells of insulin-treated diabetic animals resembled those of the normal ones (Fig. 5); the large accumulation of lipid drops was almost completely reversed with only some small droplets remaining at the basal part of the cells (Fig. 5). In contrast to the large accumulation of lipid droplets found in acinar cells of diabetic animals, the endocrine cells remaining in the islets of Langerhans of these animals (mainly glucagon, somatostatin and pancreatic polypeptide cells) (Fig. 6), as well as the epithelial cells of the exocrine ducts (Fig. 7) were devoid of lipid accumulation. These results suggest that only the acinar parenchyma is affected by the lipid changes described above.

The electron microscopy results corroborated the biochemical findings. Indeed, the cytochemical study demonstrated that the lipid droplets, present in acinar cells of diabetic animals, are of cholesterol nature, because the selective technique used yielded a positive staining (Fig. 8).

DISCUSSION

The use of animal models displaying diabetes has contributed greatly to our understanding of the disease in humans. In this study, the characteristic metabolic changes occurring in the diabetic state were noted, i.e., hyperglycemia, hyperlipidemia and hypoinsulinemia. Our



FIGS. 6 and 7. Pancreatic tissue from a diabetic animal. The endocrine cells (End) of the islet of Langerhans (Fig. 6) as well as the epithelial cells (Ep) of the secretory duct (Fig. 7) are devoid of lipid droplets. In contrast, the neighboring acinar cells display lipid accumulation. Fig. 6 ×4200. Fig. 7 ×3500.

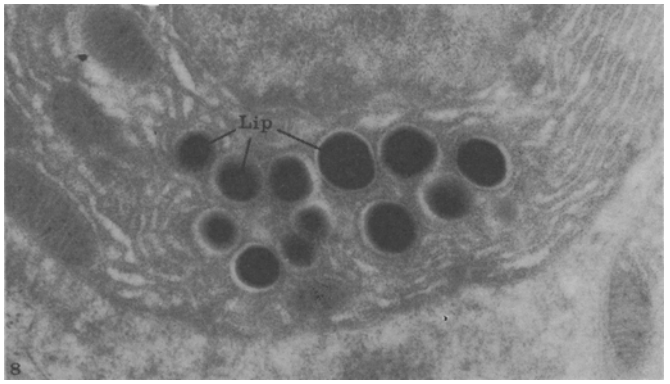


FIG. 8. Pancreatic tissue from a diabetic animal. The tissue was prepared for the demonstration of cholesterol. The lipid droplets (Lip) present in the acinar cells display a positive cytochemical reaction since they appear electron dense. ×13000.

results conclusively demonstrated that diabetes in the rat led to several changes in the concentration as well as in the composition of fatty acids and lipid components in the pancreas. The lipid classes responsible for the large diminution of total fat included TG, DG, MG and FFA. By contrast, esterified- and free-cholesterol were surprisingly high. This finding involved only acinar cells because the remaining endocrine cells as well as the epithelial cells did not show any accumulation of lipid droplets. Another crucial observation was that the phospholipids of the pancreas of experimental diabetic rats disclosed increased linoleic acid (18:2ω6) and diminished palmitoleic, oleic and arachidonic acid. Daily insulin replacement for 10 days normalized or overcorrected the abnormal lipid profile of the pancreas of diabetic rats in conjunction with restoration of the plasma changes.

At this time, the precise mechanism for the accumulation of cholesterol in the diabetic state is not known because cholesterol levels in all cells represent a balance between cellular uptake, de novo synthesis and catabolism (27). Some investigators have recently documented enhanced cholesterol synthesis in the small intestine of streptozotocin-treated rats (28,29). However, we must be careful not to extrapolate these findings to the pancreas because the activity of the key synthetic enzyme, HMG-CoA reductase, is reportedly depressed in the liver of streptozotocin-diabetic rats (30). Additional studies are required to explore the possibility of an increased uptake of cholesterol by the pancreas from circulating lipoproteins. It is evident from many studies that the major way by which numerous peripheral tissues in man may acquire cholesterol to supplement endogenous synthesis is by means of cholesterol uptake from low density lipoproteins (LDL) (31). But in animal species, such as the rat where plasma LDL levels are excessively low, HDL may fulfill this role (32). This process could be active in diabetic rats because cholesterol metabolism is altered and leads to a marked increase in plasma.

As stated before, the extensive lipid alterations found in diabetes appear to be focused only on the exocrine parenchyma. Previous studies described alterations in the secretion of amylase (4–9,13), a reduction of the exocrine mass and diminished pancreatic reserve (33,34). Others not only observed insulinitis but also severe atrophy of the acinar cells surrounding the islets of Langerhans (35). Furthermore, the pancreas of diabetic animals showed lymphatic infiltration and acinar cell damage around the islets (8). In our diabetic model, a drastic decrease in insulin secreting cells has been found in agreement with previous results (13,36). The remaining islets of Langerhans consisted mainly of glucagon- and somatostatin-secreting cells. On the other hand, the acinar parenchyma does not appear to be altered apart from changes in the secretory activity of the cells (9,13) and the presently described lipid deposition. Esters of some saturates, monounsaturates and of arachidonic acid were decreased more than esters of linoleic acid. Because of these variable individual changes, the relative composition of the total and PL fractions differed from that of controls. Benjamin and Gellhorn (37), studying adipose tissue, Brenner et al. (38), working with adipose tissue and testis, and Holman et al. (19), examining heart, liver, kidney and aorta described the same trend in the fatty acid profiles. Therefore, it seems that almost all organs are affected by the metabolic derangements of diabetes.

In view of the percent increase of linoleic acid in the pancreas of diabetic rats coupled with the substantial decrease of arachidonic acid, it is reasonable to suggest that the metabolism of linoleate is impaired. Similarly, although the relative proportion of α-linolenic acid (18:3ω3) was unchanged, the metabolite, 20:5ω3, was significantly decreased. The enzyme is responsible for the desaturation of 20:3ω6 to 20:4ω6 as well of 20:4ω3 to 20:5ω3, and of 20:2ω9 to 20:3ω9. Given that the content of the 20:3ω6 substrate for Δ5-desaturation, was found to be increased by 84% above the control value, and that arachidonic acid was suppressed by 91.9%, the activity of the n3 Δ5-desaturation enzyme must be impaired in diabetes. We have paid particular attention to the PL fraction because assessment of EFA status through PL analysis is thought to be more reliable (39). The rats in our investigation had been diabetic for more than 90 days and demonstrated a decrease total ω3 acids from 1.72 ± 0.18 in controls to 0.52 ± 0.08. Although Holman et al. (19) proposed that the magnitude and direction of the effect of diabetes on the proportions of ω3 acids is mediated

by diet, this does not seem to be the case in the present study because the diet remained identical.

Previous studies in diabetic animals reported altered physical properties and fluidity of membranes in tissues other than the pancreas (22). Our results relative to TC/PL ratios referred to the whole cell content. Therefore, additional studies are required to determine the membrane lipid composition and fluidity, and to assess their implication in the various alterations observed in diabetic pancreas.

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REFERENCES

1. Domschke, W., Tympner, F., Domschke, S., and Demling, L. (1975) *Dig. Dis. 20,* 309-312.
2. Chey, W.Y., Shay, H., and Shuman, C.R. (1963) *Ann. Intern. Med. 59,* 812-821.
3. Vacca, J.B., Henke, W.J., and Knight, W.A. (1964) *Am. Intern. Med. 61,* 242-247.
4. Korc, M., Iwamoto, Y., Sankaran, H., Williams, J.A., and Goldfine, I.D. (1981) *Am. J. Physiol. 240,* G56-G62.
5. Korc, M., Owerbach, D., Quinto, C., and Rutter, W.J. (1981) *Science 213,* 351-353.
6. Soling, H.D., and Unger, K.O. (1972) *Eur. J. Clin. Invest. 2,* 199-212.
7. Gregoire, S., and Bendayan, M. (1986) *Diabetologia 29,* 655-660.
8. Bendayan, M., Ito, S., and Manocchio, I. (1982) *Diabetologia 23,* 65-68.
9. Williams, J.A., and Godfine, I.D. (1986) *Diabetes 34,* 980-986.
10. Balk, M.V., Max Lang, C., White, W.J., and Munger, B.L. (1975) *Lab. Invest. 32,* 28-32.
11. Seemayer, T.A., Colle, E., Tannenbaum, G.S., Oligny, L.L., Guttman, R.D., and Goldman, H. (1983) *Metabolism 32,* 26-32.
12. Gepts, W., and De Mey, J. (1978) *Diabetes 27* (Suppl. 1), 251-261.
13. Bendayan, M., and Gregoire, S. (1987) *Pancreas 2,* 272-282.
14. Henderson, J.R. (1969) *Lancet 2,* 469-470.
15. Hellerstrom, C., Hellman, B., Bralin, S., and Larsson, S. (1962) *Acta Pathol. Microbiol. Scand. 54,* 1-7.
16. Bierman, E.L., Bagdade, J., and Porte, D. Jr. (1966) *Trans. Assoc. Am. Phys. 79,* 348-360.
17. Reaven, E.P., and Reaven, G.M. (1974) *J. Clin. Invest. 54,* 1167-1178.
18. Schrade, W., Boehle, E., Biegler, R., and Harmuth, E. (1962) *Lancet 2,* 285-290.
19. Holman, R.T., Johnson, S.B., Gerrard, J.M., Mauer, S.M., Kupcho-Sandberg, S., and Brown, D.M. (1983) *Proc. Natl. Acad. Sci. USA 80,* 2375-2379.
20. Peluffo, R.O., Ayala, S., and Brenner, R.R. (1970) *Am. J. Physiol. 218,* 669-673.
21. Brasitus, T.A., and Dudeja, P.K. (1985) *J. Biol. Chem. 260,* 12405-12409.
22. Anderson, L.C., and Garret, J.R. (1986) *Arch. Oral Biol. 31,* 469-475.
23. Folch, J., Lees, M., and Sloane Stanely, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
24. Lepage, G., and Roy, C.C. (1986) *J. Lipid Res. 27,* 114-120.
25. Frühling, J., Penasse, W., Sand, G., and Claude, A. (1969) *J. Microscop. 8,* 857-982.
26. Bancroft, T.A. (1968) in *Topics in Intermediate Statistical Methods,* 1st edn., The Iowa State University Press, Ames, IA.
27. Rudney, H., and Sexton, R.C. (1986) *Ann. Rev. Nutr. 6,* 245-272.
28. Feingold, K.R., Wiley, N.H., MacRae, G., Moser, A.H., Lear, S.D., and Siperstein, M.D. (1982) *Diabetes 31,* 388-395.
29. Feingold, K.R., and Moser, A.H. (1984) *Am. J. Physiol.,* G494-G501.
30. Young, N.L., Saudek, C.D., and Crawford, S.A. (1982) *J. Lipid Res. 23,* 266-275.
31. Grundy, S.M. (1984) *J. Lipid Res. 25,* 1611-1618.
32. Chen, Y.D.I., Kraemer, F.B., and Reaven, G.M. (1980) *J. Biol. Chem. 255,* 9162-9167.
33. Gepts, W. (1965) *Diabetes 14,* 619-633.
34. Dandona, P., Freedman, D.B., Foo, Y., Perkins, J., Katrak, A., Mikhailidis, D.P., Rosalki, S.B., and Beckett, A.G. (1984) *J. Clin. Pathol. 37,* 302-306.
35. Foulis, A.K., and Stewart, J.A. (1984) *Diabetologia 26,* 456-461.
36. Orci, L. (1976) in *Diabetes Research Today,* pp. 135-151, Schaltaner Verlag, New York.
37. Benjamin, W., and Gellhorn, A. (1966) *J. Lipid Res. 7,* 285-294.
38. Brenner, R.R., Peluffo, R.O., Mercuri, O., and Restelli, M.A. (1968) *Am. J. Physiol. 215,* 63-70.
39. Holman, R.T., Johnson, S.B., and Hatch, T.F. (1982) *Am. J. Clin. Nutr. 35,* 617-623.

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Formation of Trimers of α-Tocopherol and Its Model Compound, 2,2,5,7,8-Pentamethylchroman-6-ol, in Autoxidizing Methyl Linoleate

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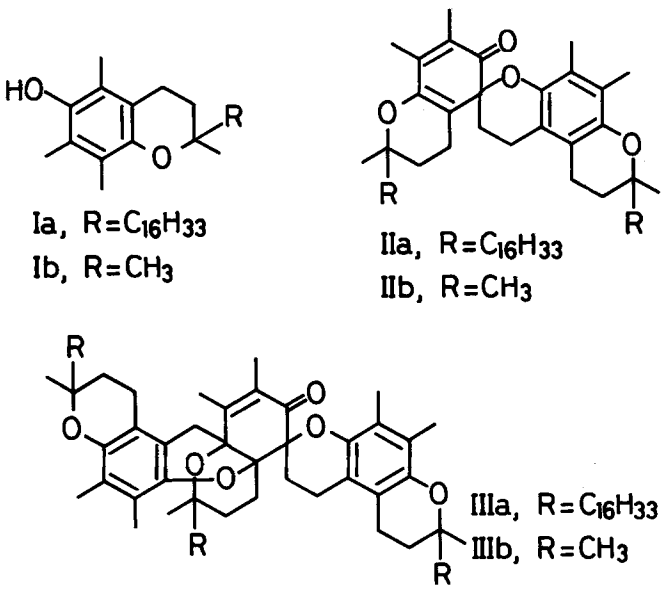
The reaction products of α-tocopherol and its model compound, 2,2,5,7,8-pentamethylchroman-6-ol, during the autoxidation of methyl linoleate at 37 C were investigated. Two isomeric trimers were obtained as the major reaction products of α-tocopherol, and a trimer was obtained as that of its model chroman. The structure of each trimer has been characterized by IR, UV, ^1H and ^{13}C NMR, and mass spectroscopy. The ^{13}C NMR spectra showed the presence of a quaternary carbon atom and a carbon atom bearing two oxy substituents in the molecule. On methanolysis of each trimer, equimolar amounts of the 5-methoxymethyl compound and the dihydroxy dimer were formed, indicating the presence of a ketal group in the molecule. From these results, new trimeric structures are proposed. The reaction products of α-tocopherol could be well-separated by reverse-phase high performance liquid chromatography. When methyl linoleate was autoxidized in the presence of 1 mol% α-tocopherol, spirodiene dimer was the initial reaction product of α-tocopherol, and trimers were formed with the decrease of α-tocopherol.
Lipids 23, 779–783 (1988).



SCHEME 1

Tocopherols are generally present in seed oils and are viewed as natural antioxidants. The role of α-tocopherol, the major constituent of vitamin E, in foods or living cells is believed to be as a free radical scavenger. Its oxidation in these systems is most likely a result of its reaction with lipid peroxy radicals (1,2). Csallany et al. (3) have shown that the major reaction products of α-tocopherol formed during autoxidation of methyl linoleate at 60 C are identical to compounds produced by mild oxidation of α-tocopherol with alkaline $K_3Fe(CN)_6$. The major products of these oxidations are the spirodiene dimer (IIa) and trimer (IIIa) of α-tocopherol (Ia) (Scheme 1). It has been well-established that free radical oxidation of α-tocopherol leading to characteristic products takes place via the tocopheroxy radical intermediate (4,5). If a suitable free-radical is not present, dimer and trimer would be expected to be major products of these reactions.

We have studied the reaction products of α-tocopherol and its model compound, 2,2,5,7,8-pentamethylchroman-6-ol (PMC), during the autoxidation of methyl linoleate in bulk phase at 37 C.

MATERIALS AND METHODS

Materials. 2*R*,4′*R*,8′*R*-α-Tocopherol (Type V) was purchased from Sigma Chemical Co. (St. Louis, MO), and 2*R*,4′*R*,8′*R*-γ-tocopherol was prepared from mixed isomers of tocopherol (Eisai Co., Tokyo, Japan). Tocopherols were purified by Sephadex LH-20 column chromatography (6).

An α-tocopherol model compound, PMC, was prepared by the method of Nilsson et al. (7), and obtained as colorless needles: mp 96–97 C; MS *m/z* 220 (M^+, 100%); IR (KBr) ν 3250 cm^{-1}; UV (hexane) λ 295 nm (ε 3420). α-Tocopheryl quinone was produced from α-tocopherol by oxidation with $FeCl_3$ in ethanol (4), while α-tocopherol spirodiene dimer was synthesized following the procedure of Nelan and Robeson (8). Methyl linoleate purchased from Tokyo Kasei Kogyo (Tokyo, Japan) was purified by silica gel column chromatography to be peroxide-free (9). All other chemicals were obtained from common laboratory suppliers. All solvents were distilled in an all-glass still before use.

High performance liquid chromatography (HPLC). HPLC was performed with a Jasco Trirotar V pump equipped with a Model GP-A40 gradient programmer and with a Model 875 UV detector. Reverse-phase HPLC was done with a Wakosil 5C18 column (4.6 × 250 mm, Wako Pure Chemical Ind., Osaka, Japan) developed with a 15 min linear gradient of methanol to methanol/isopropyl ether (1:1, v/v) at a flow rate of 1.5 ml/min. Normal-phase HPLC was done with a μBondasphere 5μ Si-100Å column (3.9 × 150 mm, Nihon Waters Ltd., Tokyo, Japan) developed with hexane/isopropyl ether (97:3, v/v) at a flow rate of 1.5 ml/min.

Oxidation procedure. For the structural analysis, methyl linoleate (3.0 g) with α-tocopherol (1.0 g) or PMC (0.60 g) was placed in a glass shalet (7 cm in diameter). To assure that the methyl linoleate and the tocopherol were well mixed, they were dissolved in ethyl ether and then dried under nitrogen and allowed to autoxidize at 37 C for 30 days in the dark. The reaction products of α-tocopherol or PMC were analyzed by thin layer chromatography (TLC) on a Merck pre-coated silica gel 60 plate

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Abbreviations: PMC, 2,2,5,7,8-pentamethylchroman-6-ol; HPLC, high performance liquid chromatography; IR, infrared; NMR, nuclear magnetic resonance; TLC, thin layer chromatography.

(Darmstadt, West Germany) using hexane/ethyl ether/acetic acid (80:30:1, v/v/v) as the solvent system. The products were separated by silica gel column chromatography. The column was developed with increasing proportions of ethyl ether in hexane. For the time-course experiment, methyl linoleate (1 mmol), either with or without 1 mol% of α-tocopherol, was placed in a glass vial (1.5 cm in diameter) and autoxidized at 37 C in the dark. At regular intervals, aliquots of the sample were withdrawn, dissolved in isopropanol and injected into the HPLC. The amounts of α-tocopherol and its reaction products were calculated from the peak area by monitoring the elutions at 285 nm. γ-Tocopherol was used as the internal standard (10). The peroxide values were measured by the iodometric method (9).

Methanolysis of the reaction products. Each solution of the products in benzene (10 ml) and 5% HCl in methanol (5 ml) was heated at 100 C for three hr in a sealed tube. After cooling, each product was extracted with ethyl ether, washed with distilled water and dried in vacuo. The extracts were analyzed by TLC with hexane/ethyl ether/acetic acid (90:10:0.5, v/v/v) as the solvent system. The methanolized products were separated by silica gel column chromatography developed with increasing proportions of ethyl ether in hexane.

Apparatus. All melting points were determined with a Yanagimoto microapparatus and are uncorrected. Proton (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-GX-270 FT NMR spectrometer with $CDCl_3$ as the solvent and with tetramethylsilane as the internal standard. NMR spectra are taken at ca. 27 C; ^{13}C NMR at 67.8 MHz with proton decoupling, and ^{1}H NMR at 270.05 MHz. Mass (MS) spectra were obtained with a Hitachi RMU-6 mass spectrometer at ionizing energy of 70 eV. Infrared (IR) spectra of samples in KBr tablets or liquid film were taken on a Jasco A-302 IR spectrometer. Ultraviolet (UV) spectra were measured with a Hitachi Model 200-10 spectrophotometer.

RESULTS

Characterization of reaction products. The reaction products of α-tocopherol in the autoxidized methyl linoleate were analyzed by TLC. Spots corresponding to two reaction products of α-tocopherol (IVa: R_f 0.96 and IVb: R_f 0.91), methyl linoleate (R_f 0.76), α-tocopherol (R_f 0.55) and methyl linoleate monohydroperoxide (the autoxidation product of methyl linoleate (11): R_f 0.26) were observed on the TLC plate. The reaction products, IVa and IVb, were separated by silica gel column chromatography and obtained as pale-yellow waxes (the yields of IVa and IVb were 106 mg and 94 mg, respectively).

The autoxidation of methyl linoleate in the presence of PMC gave methyl linoleate (R_f 0.76), a reaction product of PMC (V: R_f 0.69), PMC (R_f 0.44) and methyl linoleate monohydroperoxide (R_f 0.26) on the TLC plate. The reaction product, V, was separated by silica gel column chromatography, crystallized from ethanol/ethyl ether/water and obtained as white powder (yield 62.6 mg, mp 223–226 C).

The compounds IVa, IVb and V agreed spectrometrically with trimers of α-tocopherol and PMC (12,13). IVa: MS *m/z* 858 ([M-428]$^+$, 0.4%); IR (film) ν 1695 cm^{-1}; UV (hexane) λ 293 nm (ε 6100). IVb: MS *m/z* 858 ([M-428]$^+$, 0.1%); IR (film) ν 1690 cm^{-1}; UV (hexane) λ 293 nm (ε 6200). V: MS *m/z* 654 (M$^+$, 2%); IR (KBr) ν 1690 cm^{-1}; UV (hexane) λ 292 nm (ε 6400).

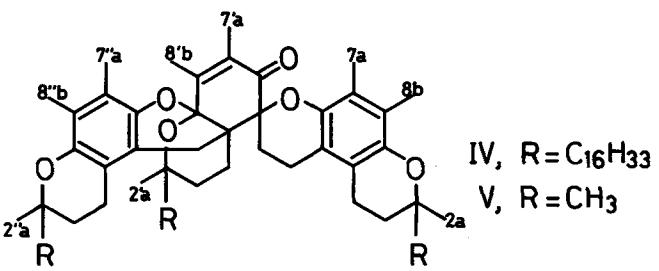
The ^{1}H NMR spectra of IVa, IVb and V were consistent with that expected for trimers of α-tocopherol and PMC (Table 1). In particular, the geometric relationship between IVa and IVb could be established by interpretation of the spectra (14,15). When the third α-tocopherol residue is *trans* to the 2′a-methyl group (IVa), the 2′a-methyl group (1.22 ppm) is localized in a shielding environment above the plane of the enone system. When the third α-tocopherol residue is *cis* to the 2′a-methyl group (IVb), deshielding of the 2′a-methyl group (1.44 ppm) is observed and may be due to 1,3-diaxial interaction of this group with the third α-tocopherol.

The ^{13}C NMR spectra of IVa, IVb and V show, in addition to other peaks that are consistent with the assigned structure as shown in Table 2, a single carbonyl (IVa: 198.9 ppm, IVb: 198.7 ppm, V: 198.7 ppm), a quaternary carbon (IVa: 42.8 ppm, IVb: 41.6 ppm, V: 41.7 ppm), four tertiary ethers (IVa: 85.6, 77.2, 74.7, and 74.3 ppm; IVb: 85.5, 76.2, 74.7, and 74.5 ppm; V: 85.5, 74.5, 72.7, and 72.4 ppm), and a carbon atom bearing the two oxy substituents (IVa: 100.3 ppm, IVb: 99.5 ppm, V: 99.7 ppm).

The trimers (IVa, IVb and V) were hydrolyzed by HCl according to the conditions of Skinner and Parkhurst (13).

TABLE 1

^{1}H NMR Chemical Shifts of the Methyl Groups of Trimers, IVa, IVb and V

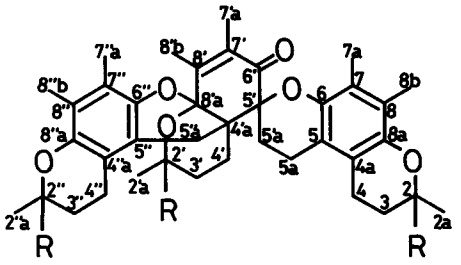


IVa	IVb	V	Assignment of methyl proton
2.23(3)[a,b]	2.23(3)	2.23(3)	
2.19(3)	2.19(3)	2.18(3)	
2.13(3)	2.13(3)	2.13(3)	7a, 8b, 7″a, 8″b
2.09(3)	2.09(3)	2.09(3)	
1.99(3)	1.96(3)	1.96(3)	
1.67(3)	1.67(3)	1.68(3)	7′a, 8′b
1.22(3)	1.44(3)	1.49(3)	2′a
1.26(3)	1.26(3)		
1.19(3)	1.19(3)		2a, 2″a
		1.32(3)	
		1.30(3)	
		1.27(6)	2a, 2b, 2′b, 2″a, 2″b
		1.24(3)	
0.88(18)	0.88(18)		
0.85(18)	0.85(18)		R

[a]Shifts in parts per million downfield relative to tetramethylsilane.
[b]Number of protons in parentheses.

TABLE 2

^{13}C NMR Chemical Shifts of Trimers, IVa, IVb and V



IVa	IVb	V	Carbon assignment
198.9[a]	198.7	198.7	6′
150.1	150.6	150.3	8′
145.8, 145.6, 144.8, 142.6	147.7, 145.6, 144.7, 142.1	145.8, 145.7, 144.9, 142.3	6, 8a, 6″, 8″a
129.8	129.4	129.7	7′
123.6(2)[b], 122.3, 122.0	123.6(2), 122.3, 122.0	123.6, 123.5, 122.3, 122.0	5, 7, 5″, 7″
115.8, 115.7, 115.5, 115.1	115.8, 115.6, 115.2, 115.1	115.6(2), 115.1, 115.0	4a, 8, 4″a, 8″
100.3	99.5	99.7	8′a
85.6	85.5	85.5	5′
77.2	76.2	74.5	2′
74.7, 74.3	74.7, 74.5	72.7, 72.4	2, 2″
42.8	41.6	41.7	4′a
44.2-11.7	44.9-11.7	33.7-11.7	2a, 3, 4, 5a, 7a, 8b, 2′a, 3′, 4′, 5′a, 7′a, 8′b 2″a, 3″, 4″, 5″a, 7″a, 8″b, R

[a]Shifts in parts per million downfield relative to tetramethylsilane.
[b]Number of carbons in parentheses.

Each hydrolyzed sample examined by TLC showed only one spot identical to that of each original trimer.

The trimers (IVa: 50.4 mg, IVb: 34.2 mg, and V: 23.0 mg, respectively) in benzene solutions were methanolized with 5% HCl in methanol. IVa and IVb gave the same two products, VIa (R_f 0.29) and VIIa (R_f 0.22), in addition to the unreacted trimer (IVa: R_f 0.76 and IVb: R_f 0.51) on the TLC plate. V (R_f 0.33) also gave two products, VIb (R_f 0.16) and VIIb (R_f 0.12) on the plate. The products were separated by silica gel column chromatography. Both VIa and VIIa were obtained as pale yellow oils (the yields of VIa and VIIa were 6.8 mg and 13.7 mg from IVa, and 4.9 mg and 10.7 mg from IVb, respectively). VIb was crystallized from hexane and obtained as white needles (yield 3.2 mg, mp 53–54 C), and VIIb from ethanol/water gave white plates (yield 7.9 mg, mp 187–188 C).

The structures of VIa, VIIa, VIb and VIIb were confirmed from their spectral data and identified as follows: VIa, 5-methoxymethyl-7,8-dimethyltocol: MS *m/z* 460 (M^+, 3%); IR (film) ν 3420 cm^{-1} (OH); UV (hexane) λ 298 nm (ε 3800); ^{1}H NMR ($CDCl_3$) δ 4.64 ppm (*s*, H), 3.42 (*s*, 3H), 2.59 (*t*, 2H, J = 6.8 Hz), 2.10 (*s*, 3H), 1.75 (*q*, 2H, J = 10.6 Hz), 1.52 (*t*, 2H, J = 6.6 Hz), 1.21 (*s*, 3H), 0.88 (*s*, 6H), 0.85 (*s*, 6H); ^{13}C NMR ($CDCl_3$) δ 147.4 ppm, 144.7, 125.8, 123.1, 115.9, 115.4, 74.4, 69.8, 58.1, 39.8, 39.4, 37.5, 37.4 (2 atoms), 37.3, 32.8, 32.7, 31.5, 28.0, 24.8, 24.5, 23.7, 22.7, 22.6 (2 atoms), 21.0, 19.9, 19.7, 11.9, 11.7. VIIa, dihydroxy dimer of α-tocopherol. The spectral data were essentially identical with the literature values (8,12): MS *m/z* 858 (M^+, 22%); IR (film) ν 3450 cm^{-1}; UV (hexane) λ 297 nm (ε 7500); ^{1}H NMR ($CDCl_3$) δ 5.68 ppm (*s*, 2H), 2.72 (broad, 4H), 2.14 (*s*, 6H), 2.11 (*s*, 6H), 1.80 (*q*, 4H, J = 6.6 Hz), 1.23 (*s*, 6H), 0.88 (*s*, 12H), 0.85 (*s*, 12H); ^{13}C NMR ($CDCl_3$) δ 145.7 ppm, 144.9, 123.4, 123.0, 121.2, 116.3, 74.6, 40.1, 39.4, 37.5, 32.8, 31.7, 28.0, 26.1, 24.8, 24.5, 23.9, 22.8, 21.7, 19.8, 12.2, 11.9. VIb, 5-methoxymethyl-2,2,7,8-tetramethylchroman-6-ol: MS *m/z* 250 (M^+, 76%); IR (KBr) ν 3320 cm^{-1} (OH); UV (hexane) λ 293 nm (ε 3700); ^{1}H NMR ($CDCl_3$) δ 4.62 ppm (*s*, H), 3.46 (*s*, 3H), 2.58 (*t*, 2H, J = 7.0 Hz), 2.13 (*s*, 3H), 2.10 (*s*, 3H), 2.09 (broad, 2H), 1.73 (*t*, 2H, J = 7.0 Hz), 1.26 (*s*, 6H); ^{13}C NMR ($CDCl_3$) δ 147.3 ppm, 144.8, 125.6, 123.0, 116.0, 115.1, 72.3, 69.6, 57.9, 32.8, 26.6, 26.5, 20.1, 11.8, 11.7. VIIb, dihydroxy dimer of PMC. The spectral data were identical with the literature values (12): MS *m/z* 438 (M_+, 74%);

IR (KBr) ν 3420 cm^{-1}; UV (hexane) λ 293 nm (ε 6600); ^1H NMR ($CDCl_3$) δ 5.61 ppm (broad *s*, 2H), 2.75 (*t*, 4H, J = 6.6 Hz), 2.73 (*s*, 4H), 2.15 (*s*, 6H), 2.11 (*s*, 6H), 1.81 (*t*, 4H, J = 6.9 Hz), 1.30 (*s*, 12H); ^{13}C NMR ($CDCl_3$) δ 145.8 ppm, 144.9, 123.3, 122.9, 121.6, 116.1, 72.6, 33.1, 26.8, 26.7, 26.0, 20.4, 12.2, 12.1, 11.9.

From above results, we propose new trimeric structures, IVa, IVb and V (Scheme 2).

Reaction products of α-tocopherol in autoxidizing methyl linoleate. Methyl linoleate was autoxidized at 37 C in the presence of 1 mol% α-tocopherol. The reaction products of α-tocopherol were analyzed by reverse-phase HPLC (Fig. 1A). Peaks corresponding to γ-tocopherol (an internal standard), α-tocopherol and the products, spirodiene dimer, an unknown peak (could not be identified) and trimer, appeared on the chromatogram. The peak corresponding to trimer was fractionated and analyzed by the normal-phase HPLC. Equal amounts of geometric isomers, IVa and IVb, appeared on the chromatogram (Fig. 1B).

Figure 2 shows the results of autoxidation of methyl linoleate with or without 1 mol% of α-tocopherol in a period of 34 days. Methyl linoleate containing 1 mol% α-tocopherol showed a linear increase in peroxides with incubation time, and α-tocopherol in methyl linoleate decreased. No decrease was observed in the level of α-tocopherol when it was mixed with methyl myristate. A small amount of spirodiene dimer formed initially, and then trimer accumulated with the decrease of α-tocopherol. No α-tocopheryl quinone, which was considered to be as the reaction product of α-tocopherol with

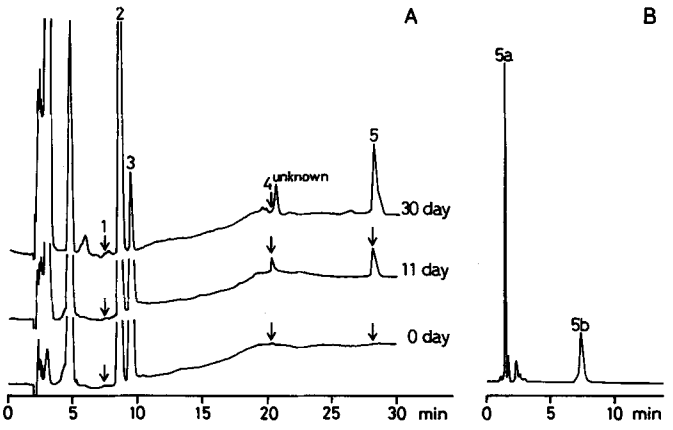
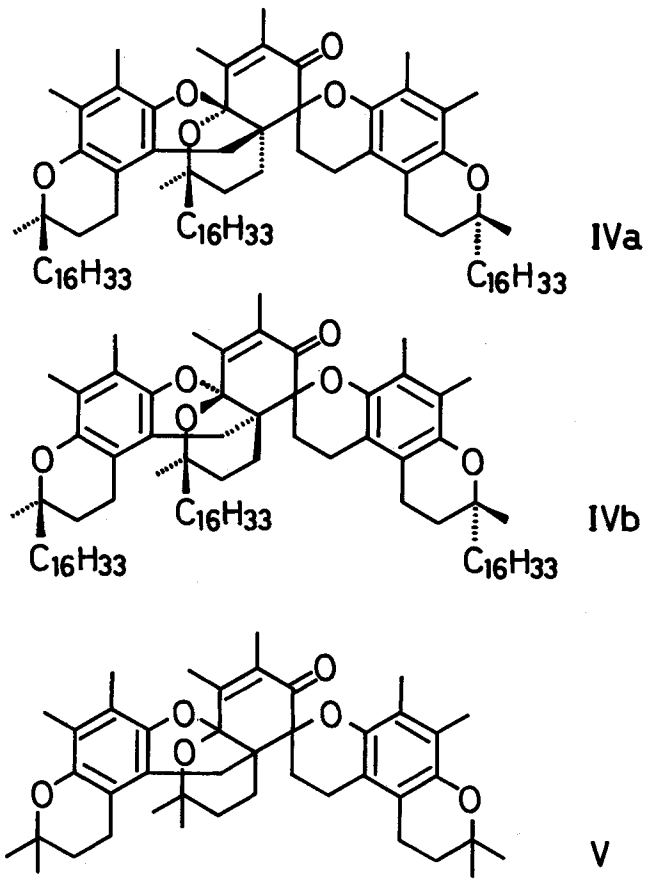


FIG. 1. Reverse-phase HPLC of the reaction products of α-tocopherol during the autoxidation of methyl linoleate for 0, 11 and 30 days (A). Arrows indicate as follows: 1, α-tocopheryl quinone; 2, γ-tocopherol (internal standard); 3, α-tocopherol; 4, spirodiene dimer; 5, trimer. Peak 5 was separated by normal-phase HPLC (B). 5a, trimer IVa; 5b, trimer IVb. The chromatographic conditions are described in the text. The eluent was monitored by an absorbance at 285 nm.



SCHEME 2

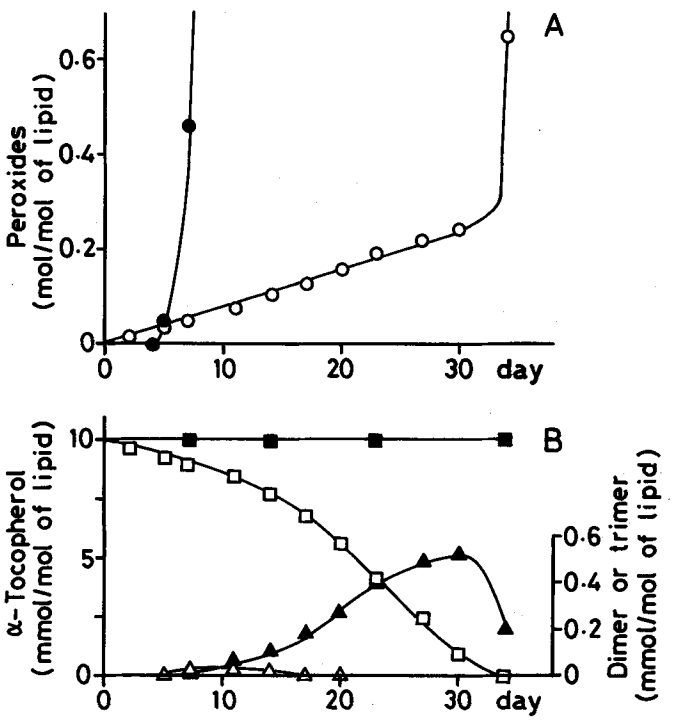


FIG. 2. Reaction of α-tocopherol during the autoxidation of methyl linoleate at 37 C. (A) Formation of total peroxides; (B) changes in amounts of α-tocopherol and its reaction products. Methyl linoleate (1 mmol) was autoxidized with 1 mol% α-tocopherol (○) and without α-tocopherol (●). Methyl myristate (1 mmol) containing 1 mol% α-tocopherol was incubated under the same conditions. Residual amounts of α-tocopherol in methyl linoleate (□) and in methyl myristate (■), and the reaction products, spirodiene dimer (△) and trimer (▲), in methyl linoleate are shown.

free radicals (16), could be detected during the reaction process.

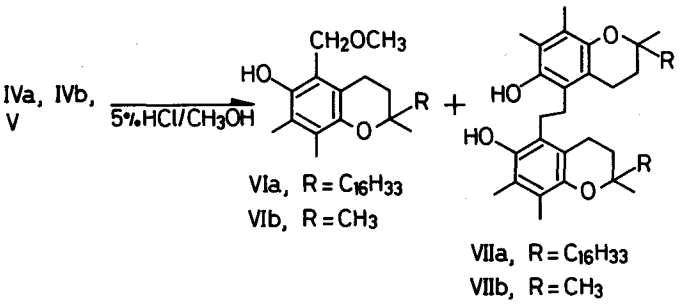
DISCUSSION

Two major products (IVa and IVb) were isolated as the reaction products of α-tocopherol formed during the autoxidation of methyl linoleate, and a major product (V) was obtained as the product of PMC oxidation. It appears that an *ortho*-quinone methide was formed from the monomer (α-tocopherol or PMC) upon oxidation by alkaline $K_3Fe(CN)_6$ (17). The *ortho*-quinone methide then added to the dimer to form the trimer. Two modes of addition of the *ortho*-quinone methide to the dimer are possible, wherein an α,β-unsaturated ketone is left in the adduct. One of those structures (III) would be acid-resistant and the others (IV and V) would be acid-labile, owing to the presence of a ketal group. Skinner and Parkhurst (13) reported the structure of trimer as III from its resistance to acid hydrolysis. The trimers reported here also were found to be acid resistant according to their experimental conditions. However, the trimers could be methanolyzed at 100 C to form equimolar quantities of the 5-methoxymethyl compound (VI) and the dihydroxy dimer (VII) (Scheme 3). Because the conditions reported by Skinner and Parkhurst were too mild for the acid hydrolysis (the sample solutions were maintained at temperature below 45 C), the trimers might be resistant toward acid. Their ^{13}C NMR spectra showed the presence of one quaternary carbon atom and one carbon atom bearing the two oxy substituents in the molecule. These results indicate that the structure of each trimer is a ketal type (Scheme 2).

The ^1H NMR spectra indicated that trimers IVa and IVb were geometric isomers. Although Skinner and Alaupovic (12) isolated two trimers of α-tocopherol (named compounds A and B and their spectral data were almost the same), they could not identify the structure of compound B. Their compounds A and B correspond to trimers IVa and IVb, respectively.

α-Tocopherol and its reaction products in the autoxidized methyl linoleate could be well separated by the reverse-phase HPLC. When methyl linoleate was autoxidized in the presence of 1 mol% α-tocopherol, the spirodiene dimer formed initially, and then the trimer accumulated. The reaction of α-tocopherol did not proceed in the saturated fatty acid methyl ester. These observations agree with those of Csallany et al. (3), who reported dimer and trimer were the major products of the autoxidation of α-tocopherol with methyl linoleate. Fujimaki et al. (4) also reported that trimer was the major product of the autoxidation of PMC after 40 days in oxygen-saturated heptane at 90 C.

α-Tocopherol at a high concentration induces the formation of lipid hydroperoxides during the autoxidation of polyunsaturated lipids (18–20). α-Tocopherol can terminate a chain reaction by donating hydrogen atoms to lipid peroxy radicals, and the resulting α-tocopheroxy



SCHEME 3

radicals participate in the prooxidant effect of α-tocopherol (20). In addition, our results indicate that the resulting α-tocopheroxy radicals react with each other and form the spirodiene dimer and trimer. The reaction products of α-tocopherol during the autoxidation of unsaturated lipids containing low levels of α-tocopherol are still unknown.

REFERENCES

1. Tappel, A.L. (1972) *Ann. N. Y. Acd. Sci. 203,* 12–28.
2. Burton, G.W., and Ingold, K.U. (1986) *Acc. Chem. Res. 19,* 194–201.
3. Csallany, A.S., Chiu, M., and Draper, H.H. (1970) *Lipids 5,* 63–70.
4. Fujimaki, M., Kanamaru, K., Kurata, T., and Igarashi, O. (1970) *Agric. Biol. Chem. 34,* 1781–1786.
5. Nilsson, J.L.G., Daves, G.D. Jr., and Folkers, K. (1968) *Acta Chem. Scand. 22,* 207–218.
6. Yamauchi, R., and Matsushita, S. (1977) *Agric. Biol. Chem. 41,* 1425–1430.
7. Nilsson, J.L.G., Sievertsson, H., and Selander, H. (1968) *Acta Chem. Scand. 22,* 3160–3170.
8. Nelan, D.R., and Robeson, C.D. (1962) *J. Am. Chem. Soc. 84,* 2963–2965.
9. Yamauchi, R., Goto, Y., Kato, K., and Ueno, Y. (1984) *Agric. Biol. Chem. 48,* 843–848.
10. Koskas, J.-P., Cillard, J., and Cillard, P. (1984) *J. Chromatogr. 287,* 442–446.
11. Frankel, E.N. (1980) *Prog. Lipid Res. 19,* 1–22.
12. Skinner, W.A., and Alaupovic, P. (1963) *J. Org. Chem. 28,* 2854–2858.
13. Skinner, W.A., and Parkhurst, R.M. (1964) *J. Org. Chem. 29,* 3601–3603.
14. Grams, G.W., Eskins, K., and Inglett, G.E. (1972) *J. Am. Chem. Soc. 94,* 866–868.
15. Yamauchi, R., and Matsushita, S. (1979) *Agric. Biol. Chem. 43,* 2151–2156.
16. Gruger, E.H. Jr., and Tappel, A.L. (1970) *Lipids 5,* 326–331.
17. Nilsson, J.L.G., Branstad, J.-O., and Sievertsson, H. (1968) *Acta Pharm. Suecica 5,* 509–516.
18. Peers, K.E., Coxon, D.T., and Chan, H.W.-S. (1981) *J. Sci. Food Agric. 32,* 898–904.
19. Koskas, P.P., Cillard, J., and Cillard, P. (1984) *J. Am. Oil Chem. Soc. 61,* 1466–1469.
20. Terao, J., and Matsushita, S. (1986) *Lipids 21,* 255–260.

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Essential Fatty Acid Pattern of Glycerolipids in Rat Hepatocytes in Primary Culture and in Coculture With Rat Liver Epithelial Cells

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The linoleic acid content of phosphatidylethanolamine (PE), phosphatidylcholine (PC) and triglyceride (TG) rapidly fell in rat hepatocytes in primary culture up to four days and in coculture with liver epithelial cells up to eight days. At the same time, the level of polyunsaturated fatty acids (PUFA), especially arachidonic acid, remained constant in PE, slightly decreased in PC and dropped in TG. There was no variation of the nonessential PUFA, 20:3n-9.

Linoleic acid supplementation of cultures 24 hr before the harvest induced a rise in the linoleic acid level of the three lipid classes. Arachidonic acid remained constant in TG and only slightly decreased in PE and PC at day 4 of primary culture and day 8 of coculture. The level of 20:3n-9 increased in PE and PC and much more in TG. This net increase in the arachidonic acid and 20:3n-9 levels in TG could not be explained only by a transfer from the phospholipid pools of PUFA because the phospholipid content of hepatocytes and PUFA levels of phospholipids did not vary under linoleic supplementation. The low percentage of arachidonic acid in epithelial cells rules out any participation of these cells in the increase of arachidonic acid in supplemented cocultures.

Triglycerides may act as a storage pool for plasma PUFA up to four days of primary culture and eight days of coculture. Besides, coculture seems more potent than primary culture to maintain the phospholipid level, to spare the essential PUFA in PE and to increase the TG synthesis in response to linoleic acid supplementation. *Lipids 23,* **784-790 (1988).**

The liver is essential to maintain homeostasis of polyunsaturated fatty acids (PUFA). In man, a decrease in the linoleic and arachidonic acid content of lipoproteins and of platelet and erythrocyte phospholipids has been shown to occur during different hepatic diseases in which hepatic function and/or biliary secretion are altered (1-4). During essential fatty acid (EFA) deprivation in the rat, Lefkowith et al. (5) observed a decrease in the linoleic acid content of all lipid classes of the liver, whereas the level of arachidonic acid decreased only in phosphatidylcholine (PC), phosphatidylinositol (PI) and neutral lipids but not in phosphatidylethanolamine (PE) or in phosphatidylserine (PS) (5).

Several authors have reported mechanisms of arachidonic acid transfer between phospholipids in thymocytes (6), macrophages (7,8) and platelets (9,10). This might explain the sparing of arachidonic acid and its accumulation in PE as shown by Aeberhard et al. in cultured skin fibroblasts (11).

We have studied the uptake of PUFA of the n-6 series by adult rat hepatocytes in primary culture (12). We have shown that PUFA were as effective as other fatty acids for triglyceride (TG) synthesis in hepatocytes. On the contrary, the incorporation into phospholipids was not related to an increase in the phospholipid content of hepatocytes and therefore must occur by the deacylation-reacylation cycle (12). From these results, the question arose of whether liver triglycerides as well as phospholipids act as a storage pool for plasma arachidonic acid.

The behavior of hepatocytes in long-term culture needed to be studied with respect to their fatty acid pattern. To maintain the phenotypic expression of hepatocytes in culture for long periods of time, Guguen-Guillouzo et al. have perfected a system in which hepatocytes are cocultured with a liver epithelial cell line (13).

In the present paper, we compared the fatty acid composition of PE, PC and TG of rat hepatocytes in primary culture up to four days with those of hepatocytes cocultured up to eight days. In addition, we studied the effect of a linoleic acid supplementation of the culture medium.

MATERIAL AND METHODS

Materials. Linoleic acid and fatty acid-free bovine serum albumin (Grade V) were obtained from Sigma Chemical Co. (St. Louis, MO). Minimum essential medium (MEM) and 199 medium were purchased from Boehringer (Mannheim, FRG).

Hepatocyte culture preparation. Hepatocytes were isolated by collagenase perfusion (14) from male adult Sprague-Dawley rats weighing 200-250 g and fed a standard chow comprised of 4% fat (w/w) with 60 mol% linoleic acid (U.A.R., France). Hepatocytes were seeded at a density of 8×10^6 cells per 75 cm^2 flask in 10 ml of the nutrient medium composed of a mixture of MEM and medium 199 (75:25, v/v) containing 80 IU/ml insulin and 10% fetal calf serum (v/v). The medium was renewed four hr after cell seeding. At that time, a part of the cultures was set up as cocultures by adding an equivalent number of rat liver epithelial cells (RLEC) (13).

RLEC were prepared from young rat livers and subcultivated in Williams medium supplemented with 10% fetal calf serum, as described elsewhere (15). For both pure culture and coculture, the medium supplemented with 7×10^{-5} M hemisuccinate hydrocortisone was renewed every day thereafter.

Pure RLEC cultures used as controls were maintained in Williams medium supplemented with serum until confluency was reached, then washed with phosphate-buffered saline and shifted to the medium used for hepatocyte cultures.

Incubation procedure. Primary cultures were carried out for one, two or four days, cocultures for four or eight

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Abbreviations: EFA, essential fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; TG, triglycerides; MEM, minimum essential medium; RLEC, rat liver epithelial cells.

days and epithelial cells for three days in a medium MEM/199 3:1 (v/v) containing insulin and hydrocortisone as indicated above and 0.15 mM fatty acid free bovine albumin prepared as described (16).

The medium was changed every day. Half of the flasks were supplemented the last 24 hr with 0.3 mM linoleic acid bound to fatty free albumin at a ratio linoleic acid/albumin = 2. At the end of the incubation, the medium was removed, the plated cells were washed, collected and disrupted as described (12). Cell samples were collected for protein assay according to Lowry et al. (17) and extracted as described by Bligh and Dyer (18).

Lipid analysis. TG, PC and PE, were separated by preparative thin layer chromatography using a two-step migration in the same direction as described (12). Samples spots were scrapped off by comparison to standards, revealed by iodine vapor. Enzymatic assay of triglycerides was directly performed on the silica gel extract (18). Lipid phosphorus was assayed as described by Chen (19). For gas liquid chromatography analysis, hydrolysis and methylation (20) were performed directly on silica gel. Fatty acid separation was achieved on a 25 m capillary column filled with 20 M Carbowax operating between 200 C and 220 C at 1 C per min. Detection was by flame ionization.

RESULTS

The triglyceride content of hepatocytes remained constant in primary culture up to four days but slightly increased in coculture after eight days (Table 1). The phospholipid content of hepatocytes decreased as a function of time in primary cultures as well as in cocultures. The addition of linoleic acid 24 hr before the harvest did not modify the phospholipid content but increased the triglyceride content of hepatocytes in all the cases (Table 1).

To assess the respective lipid content of hepatocytes and epithelial cells in coculture, we also assayed lipids in epithelial cells just before confluency (3 days of culture). The triglyceride level of epithelial cells was lower than in hepatocytes in primary culture, whereas their phospholipid content was in the same range. Because epithelial cells accounted for half the cocultured cells, and the protein weights of epithelial cells and hepatocytes were, respectively, around 0.3 and 1 mg per 10^6 cells, we could estimate that epithelial cell proteins represented 25% of

TABLE 1

Lipid Content of Cultured Cells

Supplementation with linoleic acid	Triglycerides (μg/mg protein)		Phospholipids (μg/mg protein)	
	−	+	−	+
Primary culture				
Day 0	22 ± 2		101 ± 3	
Day 1	24 ± 4	30 ± 1	94 ± 4	
Day 2	20 ± 1	52 ± 2[c]	83 ± 4[a]	88 ± 4
Day 4	28 ± 3[a]	52 ± 3[c]	80 ± 2	83 ± 2
Coculture				
Day 4	28 ± 1	52 ± 2[c]	98 ± 3[b]	103 ± 8
Day 8	35 ± 4[a]	70 ± 2[c]	85 ± 5[a]	82 ± 1
Epithelial cells				
Day 3	14 ± 1	25 ± 2[a]	106 ± 4	98 ± 2

Results are means ± SD from triplicate experiments.

[a] $p < 0.05$ and

[b] $p < 0.01$ as compared to the day before.

[c] $p < 0.01$ as compared with the linoleic nonsupplemented control. Other comparisons were not significant.

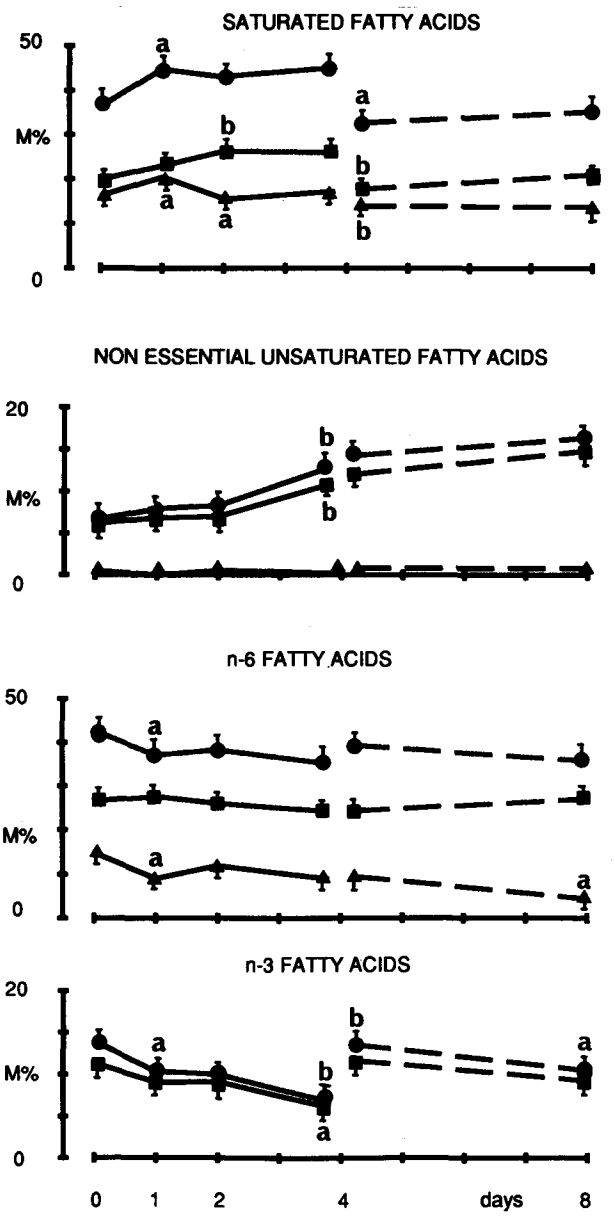

FIG. 1. Fatty acid distribution in phosphatidylethanolamine of rat hepatocytes. Primary cultures (—) were carried out for one, two and four days and cocultures (— —) for four and eight days. The standard medium was changed every day. At the indicated time, the medium was removed, the cells were washed, collected and extracted to perform GLC analysis. Results, expressed as molar percentage of each fatty acid, were means ± SD from triplicate experiments. (a) $p < 0.05$ and (b) $p < 0.01$ as compared with the day before. Total saturated fatty acids, ●; palmitic acid, ▲; stearic acid, ■. Total nonessential unsaturated fatty acids, ●; oleic acid, ■; 20:3n-9, ▲. Total n-6 fatty acids, ●; linoleic acid, ▲; arachidonic acid, ■. Total n-3 fatty acids, ●; docosahexaenoic acid, ■.

coculture proteins. The triglyceride contents of hepatocytes and epithelial cells in coculture were respectively, 24.5 and 3.5 μg per mg of coculture proteins in the flasks incubated without linoleic acid and 45.7 and 6.3 μg in the flasks supplemented with linoleic acid. We could, therefore, estimate the proper triglyceride contents of hepatocytes after four days of coculture: 33 and 61 μg/mg of hepatocyte proteins in cells incubated, respectively, without and with linoleic acid.

The fatty acid composition of PE varied slightly during either primary culture or coculture (Fig. 1). We observed only an increase in monounsaturated fatty acids and a concomitant decrease in fatty acids of the n-6 and n-3 series after four days of primary culture and after eight days of coculture (Fig. 1). More precisely, linoleic acid fell from 14.8% at day 0 to 8.9% after four days of primary culture and to 4.3% after eight days of coculture, while arachidonic acid did not change. The nonessential PUFA (20:3n-9) did not vary even after eight days of coculture.

The fatty acid variations were larger in PC (Fig. 2) than in PE. The monounsaturated fatty acid level rose with the incubation time of both primary culture and coculture. At the same time, fatty acids of the n-6 and n-3 series decreased, and in contrast with PE, this decrease affected both linoleic and arachidonic acids. These fatty acids fell, respectively, from 17.1% (linoleic acid) and 28.3% (arachidonic acid) at day 0 to 7.1% and 23.2% after four days of primary culture and to 4.4% and 20.2% after eight days

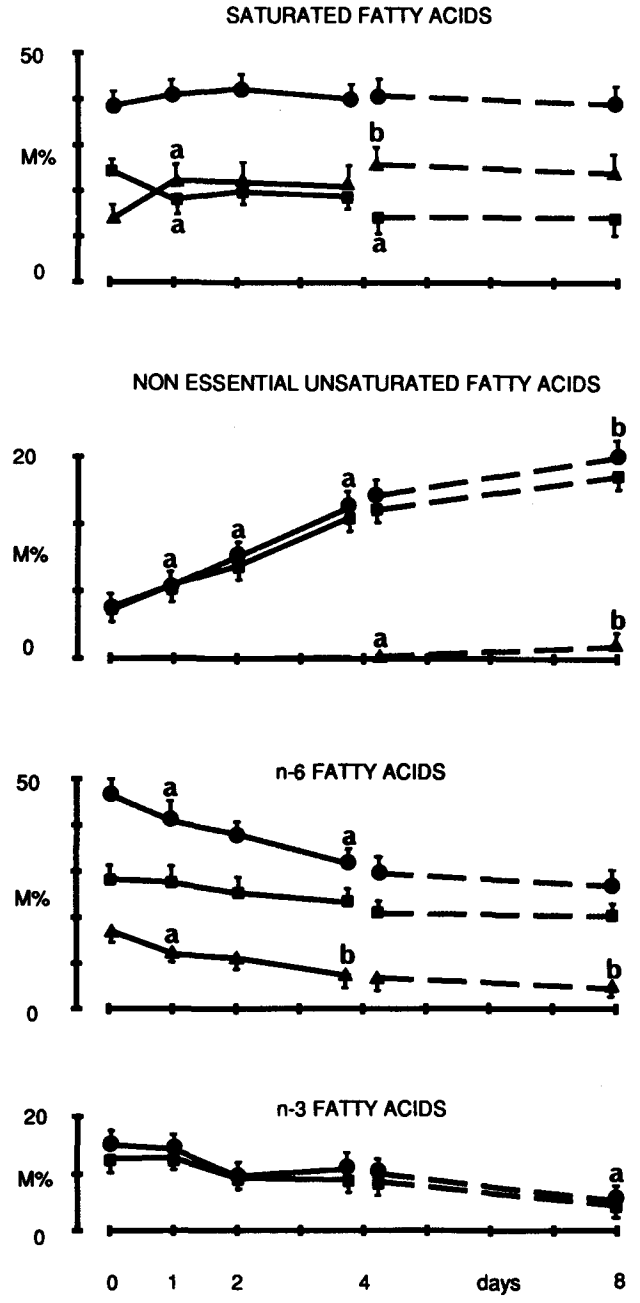

FIG. 2. Fatty acid distribution in phosphatidylcholine of rat hepatocytes. For legend, see Figure 1.

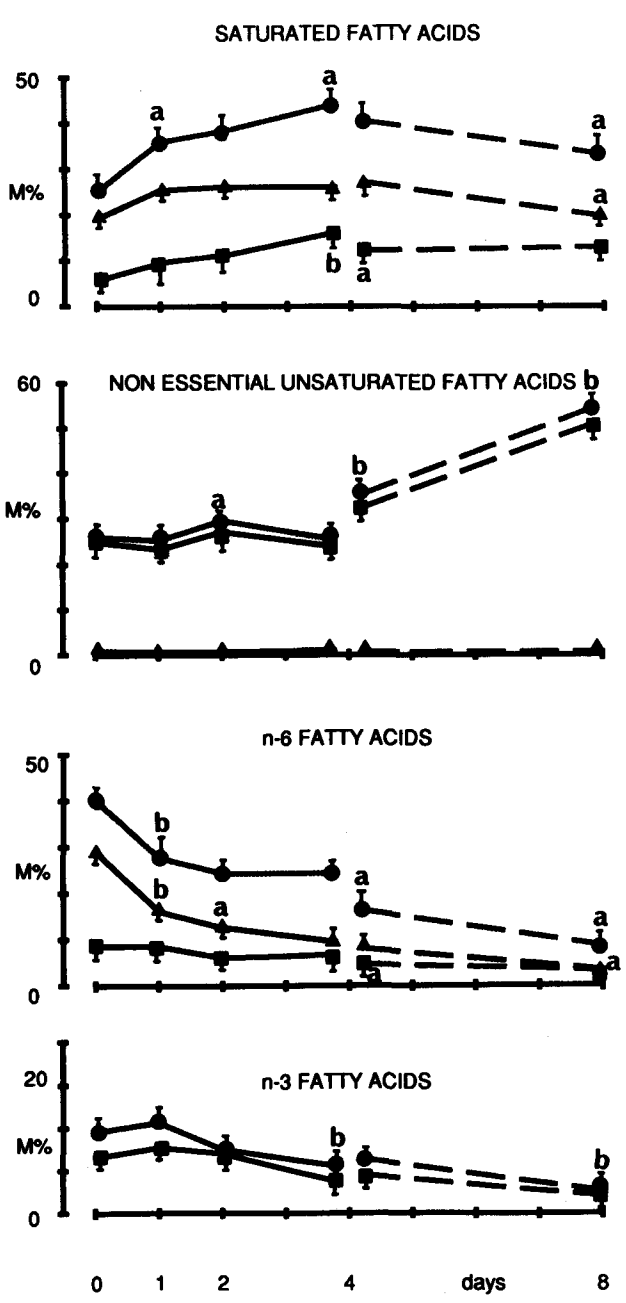

FIG. 3. Fatty acid distribution in triglycerides of rat hepatocytes. For legend, see Figure 1.

of coculture (Fig. 2). The nonessential PUFA (20:3n-9) increased only after eight days of coculture.

Figure 3 shows the dramatic loss of essential fatty acids in triglycerides. When the linoleic and arachidonic acid contents of the triglyceride fraction were respectively, 29.1% and 8.6% at day 0, it fell to 9.3% and 6.8% after four days of primary culture and to 3.3% and 2.6% after eight days of coculture (Fig. 3). The n-3 fatty acids decreased from 9.5% to 2.6% after eight days of coculture. This decrease in EFA was counterbalanced by an increase in saturated fatty acids in primary culture and by an increase in monounsaturated fatty acids in coculture. As in PC and PE, the nonessential PUFA (20:3n-9) remained low (Fig. 3).

Linoleic acid supplementation of primary culture and coculture medium 24 hr before the harvest induced a significant increase in the linoleic acid content of PE (Table 2), PC (Table 3) and TG (Table 4). The increase in linoleic acid in PE and PC was greater in primary culture than in coculture: for example, whereas linoleic acid level was in the same range in deprived primary culture and coculture at day 4, under supplementation it reached 25.2 ± 1.3 and 23.8 ± 0.1 in primary culture vs 17.2 ± 1.1 and 17.0 ± 4.0 in coculture respectively in PE and PC, whereas we observed no difference in TG. The level reached in PE, PC, and TG was nearly the same after two and four days of primary culture but higher than the level reached after 24 hr. In PE, this increase was compensated by a decrease in saturated fatty acids. The level of arachidonic acid and of n-3 fatty acids in PE remained nearly the same, except after four days of primary culture (Table 2).

In PC, the increase of linoleic acid was associated with a decrease in monounsaturated fatty acids and fatty acids of the n-3 series. The arachidonic acid content did not vary after one and two days of primary culture and four days of coculture but fell after four days of primary culture and eight days of coculture (Table 3). In both culture systems, the increase in linoleic acid in TG was paralleled by a decrease in monounsaturated and saturated fatty acids and, to a lesser extent the fatty acids of the n-3 series (Table 4). The arachidonic acid percentage in TG did not vary in primary culture but slightly increase in coculture.

Unexpectedly, we observed an increase in the nonessential PUFA (20:3n-9) level in all lipid classes of hepatocytes in primary culture and in coculture supplemented with linoleic acid. The level reached by 20.3n-9 was higher in TG (Table 4) than in PE (Table 2) and PC (Table 3), i.e., 4.0 ± 0.3, 0.75 ± 0.15 and 1.1 ± 0.1, respectively, at day 4 of primary culture.

To assess the part taken by epithelial cells in the changes that we observed with cocultures, we have performed the same experiments on epithelial cells cultured alone during three days. Table 5 displays the fatty acid composition of epithelial cells supplemented with or without linoleic acid the last 24 hr. The essential fatty acid content of PE, PC and TG was very low in cells incubated without linoleic acid, as compared with hepatocytes in primary culture. Linoleic acid supplementation induced a large enrichment in linoleic acid, which was mainly compensated in PE and PC by a decrease in monounsaturated fatty acids and in TG by a decrease in saturated fatty acids.

Arachidonic acid was not modified, whereas docosahexaenoic acid decreased slightly in PE and PC but increased in TG. We also observed in epithelial cells a surprising increase in the 20:3n-9 level in the three lipid classes.

DISCUSSION

Our results displayed a rapid fall in the linoleic acid content of rat hepatocytes in primary culture and in coculture with rat liver epithelial cells. After four days of primary culture, the linoleic acid content in PE, PC and TG was, respectively, 60, 65 and 32% of the level at day 0. It fell

TABLE 2

Effect of Linoleic Acid Supplementation on the Fatty Acid Composition of Hepatocyte Phosphatidylethanolamine

	Supplementation with linoleic acid 24 hr before harvest	Primary culture			Coculture	
		Day 1	Day 2	Day 4	Day 4	Day 8
Saturated	−	44.7 ± 2.6	43.0 ± 1.9	45.0 ± 2.3	32.6 ± 6.2	35.1 ± 10.1
	+	29.5 ± 2.3[b]	29.3 ± 1.2[b]	40.5 ± 2.5	27.6 ± 3.4	27.0 ± 2.1
Monounsaturated	−	7.9 ± 0.9	8.3 ± 0.9	12.9 ± 1.4	14.1 ± 1.9	16.2 ± 3.0
	+	9.2 ± 0.5	7.8 ± 1.0	17.0 ± 1.0[a]	11.3 ± 0.7	15.6 ± 0.5
Linoleic	−	9.0 ± 2.0	12.0 ± 0.3	8.9 ± 3.6	9.4 ± 2.3	4.3 ± 0.2
	+	22.4 ± 2.9[b]	27.1 ± 1.3[b]	25.2 ± 1.3[b]	17.2 ± 1.1[b]	13.8 ± 1.2[b]
Arachidonic	−	27.5 ± 3.4	26.0 ± 2.5	24.1 ± 0.3	24.1 ± 0.6	27.0 ± 3.9
	+	24.0 ± 1.7	21.8 ± 0.4	14.1 ± 4.7[b]	23.1 ± 2.5	25.4 ± 0.6
n-3 Fatty acids	−	10.3 ± 1.6	10.0 ± 0.5	6.8 ± 0.1	13.5 ± 0.3	10.4 ± 1.7
	+	12.2 ± 0.7	9.0 ± 1.2	4.3 ± 0.9[a]	12.7 ± 0.5	10.1 ± 1.1
Eicosatrienoic (20:3n-9)	−	ND	0.50 ± 0.25	0.15 ± 0.15	0.35 ± 0.05	0.80 ± 0.10
	+	0.50 ± 0.1	0.75 ± 0.25	0.75 ± 0.15[b]	1.40 ± 0.20[b]	1.30 ± 0.10[a]

Results, expressed as molar percentage of each fatty acid in PE, are means + SD from triplicate experiments.

[a] $p < 0.05$ and

[b] $p < 0.01$ as compared with the linoleic nonsupplemented control. Other comparisons were not significant.

TABLE 3

Effect of Linoleic Acid Supplementation on the Fatty Acid Composition of Hepatocyte Phosphatidylcholine

	Supplementation with linoleic acid 24 hr before harvest	Primary culture			Coculture	
		Day 1	Day 2	Day 4	Day 4	Day 8
Saturated	−	40.9 ± 1.8	42.2 ± 2.0	40.0 ± 2.0	40.7 ± 1.8	38.8 ± 5.0
	+	41.7 ± 2.0	41.8 ± 3.8	41.8 ± 3.8	43.0 ± 3.9	48.8 ± 3.0[a]
Monounsaturated	−	11.0 ± 1.4	15.1 ± 1.0	22.6 ± 0.2	24.2 ± 0.4	29.9 ± 0.6
	+	9.8 ± 0.9	6.3 ± 0.3[b]	12.0 ± 0.4[b]	12.9 ± 0.5[b]	20.8 ± 2.2[b]
Linoleic	−	12.0 ± 1.9	11.1 ± 0.9	7.1 ± 0.6	6.8 ± 0.4	4.4 ± 0.6
	+	17.8 ± 0.7[b]	20.1 ± 1.2[b]	23.8 ± 0.1[b]	17.0 ± 4.0[b]	15.4 ± 4.3[b]
Arachidonic	−	27.5 ± 3.1	25.8 ± 2.3	23.2 ± 2.7	20.9 ± 1.2	20.2 ± 4.0
	+	26.2 ± 2.3	25.8 ± 2.7	17.0 ± 1.2[a]	20.7 ± 0.1	11.5 ± 0.5[b]
n-3 Fatty acids	−	7.2 ± 1.6	4.8 ± 1.3	5.4 ± 0.3	5.0 ± 1.5	2.6 ± 0.7
	+	5.8 ± 1.0	3.9 ± 1.1	2.6 ± 0.2[b]	2.5 ± 0.1[b]	0.8 ± 0.1[a]
Eicosatrienoic (20:3n-9)	−	0.20 ± 0.12	ND	0.25 ± 0.05	0.40 ± 0.10	1.90 ± 0.20
	+	0.45 ± 0.05	0.70 ± 0.1[b]	1.10 ± 0.1[b]	2.10 ± 0.10[b]	1.4 ± 0.10[a]

Results, expressed as molar percentage of each fatty acid in PC, are means + SD from triplicate experiments.

[a]$p < 0.05$ and

[b]$p < 0.01$ as compared with the linoleic nonsupplemented control. Other comparisons were not significant.

TABLE 4

Effect of Linoleic Acid Supplementation on the Fatty Acid Composition of Hepatocyte Triglyceride

	Supplementation with linoleic acid 24 hr before harvest	Primary culture			Coculture	
		Day 1	Day 2	Day 4	Day 4	Day 8
Saturated	−	35.9 ± 8.0	38.3 ± 2.8	44.2 ± 0.7	40.6 ± 0.6	33.2 ± 3.1
	+	40.0 ± 3.2	26.2 ± 1.0[b]	25.0 ± 3.1[b]	22.0 ± 0.8[b]	25.7 ± 2.1[b]
Monounsaturated	−	25.2 ± 1.6	29.5 ± 0.9	25.3 ± 1.1	35.2 ± 2.7	55.1 ± 2.2
	+	22.0 ± 1.4	14.0 ± 0.4[b]	13.3 ± 0.9[b]	18.6 ± 2.4[b]	25.7 ± 2.5[b]
Linoleic	−	16.1 ± 1.7	12.6 ± 0.7	9.3 ± 2.6	8.0 ± 1.8	3.3 ± 0.1
	+	24.8 ± 3.8[a]	38.3 ± 3.5[b]	39.7 ± 2.4[b]	38.4 ± 2.4[b]	22.0 ± 2.0[b]
Arachidonic	−	8.5 ± 2.3	6.1 ± 0.6	6.8 ± 0.1	4.6 ± 0.5	3.2 ± 0.2
	+	5.4 ± 1.3	6.1 ± 0.7	6.7 ± 0.2	6.2 ± 0.4[a]	4.8 ± 0.2[b]
n-3 Fatty acids	−	10.6 ± 4.0	7.5 ± 0.3	5.5 ± 0.2	6.4 ± 0.4	2.6 ± 0.5
	+	4.3 ± 0.4[b]	6.9 ± 0.4	3.2 ± 0.2[b]	4.5 ± 0.2[b]	2.1 ± 0.3
Eicosatrienoic (20:3n-9)	−	0.58 ± 0.11	0.55 ± 0.05	0.9 ± 0.30	0.60 ± 0.0	0.6 ± 0.0
	+	0.95 ± 0.15	3.7 ± 0.5[b]	4.0 ± 0.3[b]	3.20 ± 0.70[b]	2.1 ± 0.5[b]

Results, expressed as molar percentage of each fatty acid in TG, are means + SD from triplicate experiments.

[a]$p < 0.05$ and

[b]$p < 0.01$ as compared with the linoleic nonsupplemented control. Other comparisons were not significant.

to 26%, 25% and 11%, respectively, after eight days of coculture. These results confirm those obtained by Lefkowith et al. with suckling mice deprived of essential fatty acids (5). These authors observed that the linoleic acid depletion was higher in the liver than in other tissues. They showed that it was paralleled by a progressive fall in the arachidonic acid content in rat liver PC, PI and neutral lipids but not in PE and PS.

We observed a similar evolution after four days of primary culture and eight days of coculture: arachidonic acid did not decrease in PE, decreased slightly in PC but broke down in TG. At the same time, the end-product of the n-3 series, docosahexaenoic acid, exhibited a moderate decrease in PE but a larger one in PC and TG. These data evidenced the tenacity by which the organism can conserve arachidonic acid when the diet is deprived in EFA,

TABLE 5

Fatty Acid Composition of Epithelial Cell Line Lipids after Three Days of Culture

	PE		PC		TG	
	−	+	−	+	−	+
Saturated	33.9 ± 2.1	39.2 ± 1.5[a]	49.4 ± 4.6	45.0 ± 2.7	73.9 ± 0.1	31.4 ± 0.3[b]
Monounsaturated	36.7 ± 2.1	15.7 ± 1.6[b]	41.6 ± 3.9	23.1 ± 1.9[b]	21.2 ± 1.5	20.1 ± 0.4
Linoleic	3.9 ± 0.5	15.5 ± 0.9[b]	2.8 ± 0.6	24.0 ± 0.6[b]	3.1 ± 1.2	31.7 ± 1.0[b]
Arachidonic	11.0 ± 1.7	10.8 ± 0.6	3.5 ± 0.6	3.2 ± 0.4	1.2 ± 0.3	1.7 ± 0.1
Docosahexaenoic	6.1 ± 0.7	4.6 ± 1.1	1.1 ± 0.3	0.1 ± 0.1[b]	0.7 ± 0.1	2.4 ± 0.2[b]
20:3n-9	1.3 ± 0.4[a]	2.8 ± 0.5	1.0 ± 0.2	3.5 ± 0.7[b]	1.0 ± 0.2	7.3 ± 0.5[b]

Results, expressed as molar percentage of each fatty acid in each lipid class, are means + SD from triplicate experiments.

[a]$p < 0.05$ and

[b]$p < 0.01$ as compared to the linoleic nonsupplemented control. Other comparisons were not significant.

a tenacity that was underestimated before Lefkowith's report. We further demonstrated that this tenacity involves a mechanism that is conserved up to four days of primary culture and up to eight days of coculture of rat hepatocytes.

We found that PE was more stable than PC when cultured hepatocytes were deprived of EFA, in accordance with Lefkowith et al. (5). Furthermore, after four days, cocultured hepatocytes exhibited a greater stability of PE composition than cells in primary culture. This effect could not be explained by an arachidonic acid transfer from epithelial cells to hepatocytes because epithelial cells were largely depleted in arachidonic acid (Table 5).

The fatty acyl composition of PE remained stable when hepatocytes in primary culture or in coculture were supplied with linoleic acid. This agrees with Weiner and Sprecher, who observed that feeding rats with linoleate did not significantly modify the arachidonic acid level in liver and platelet phospholipids (21). Furthermore, PC and PE exhibited a clear difference in the fate of their fatty acids when linoleic acid was added to the culture medium 24 hr before the harvest. The linoleic acid increase was balanced by a decrease of saturated fatty acid in PE (Table 2) and by a decrease in monoenoic fatty acids in PC (Table 3). These differences might be explained by a different potency of the deacylation-reacylation cycle towards PE and PC and/or by the existence of sparing mechanisms for arachidonyl molecular species of PE such as those evidenced by Aeberhard et al. in cultured skin fibroblasts (11).

The nonessential PUFA 20:3n-9 did not increase in PE or in PC during primary culture and only slightly increased in cocultures. This confirms a previous observation that, during a more severe EFA deprivation (8 wk), 20:3n-9 moderately accumulated in liver, whereas its accumulation in other tissues was much greater (5). Similarly, 20:3n-9 accumulated into 3T3 cells in culture with 10% fetal bovine serum (22).

As we have reported (12), linoleic acid addition to the medium induced an increase in the TG level but no change in the PL level of hepatocytes in primary culture as well as in coculture (Table 1). Whereas TG were highly enriched in linoleic acid, the arachidonic acid level remained rather stable, and the 20:3n-9 level increased. Taking into account the increase in the TG mass of hepatocytes in primary culture incubated with linoleic acid, we can evidence a net increase in both arachidonic acid and 20:3n-9 levels in TG: 2- to 3-fold and 7- to 17-fold, respectively (Table 6). This net increase might have been explained by a fatty acid transfer from phospholipid stores of polyunsaturated fatty acids because we have observed a phospholipid loss as a function of time. However, (1) medium was changed every day, (2) the PL content of hepatocytes remained constant under linoleic acid supplementation, and (3) arachidonic acid and 20:3n-9 levels remained constant in PL at least during two days of primary culture and four days of coculture (Tables 2 and 3).

TABLE 6

Net Increase of Arachidonic Acid or Mead Acid in Hepatocyte Triglycerides after Linoleic Acid Supplementation

	Primary culture		Coculture	
	Day 2	Day 4	Day 4	Day 8
Arachidonic	1.95 (×2.6)	1.58 (×1.8)	1.94 (×2.5)	2.24 (×3.0)
(20:3n-9)	1.81 (×17)	1.87 (×8.5)	1.50 (×9.9)	1.86 (×7.0)

Results are expressed in μg/mg of proteins. Values in parentheses are the ratio between these data and the mass of triglyceride arachidonic acid in cultures nonsupplemented with linoleic acid. This mass was calculated from the triglyceride level (Table 1) and the fatty acid composition of triglycerides (Table 4) at the indicated times of incubation.

The low percentage of arachidonic acid observed in epithelial cells cultured in presence or in absence of linoleic acid, rules out any participation of these cells in the increase of the arachidonic acid in TG of cocultures. The large increase of 20:3n-9 in TG of epithelial cells when they were supplemented with linoleic acid did not allow us to assess the respective role of hepatocytes and epithelial cells in the rise of this fatty acid in coculture. Additional experiments remain to be made to measure a putative Δ5 or Δ6 desaturase induction or stimulation under such circumstances.

Nevertheless, TG appear to act as a storage pool for plasma PUFA up to four days in primary culture and to eight days in cocultures. Besides a coculture seems more potent than primary culture to maintain the phospholipid level, to spare the essential PUFA in PE and to increase the TG synthesis in response to linoleic acid supplementation.

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REFERENCES

1. Picard, J., Veissière, D., Voyer, F., and Béréziat, G. (1972) *Clin. Chim. Acta 36,* 247-250.
2. Petit, J., Poupon, R., Trugnan, G., Béréziat, G., and Darnis, F. (1978) *Digestion 79,* 153-154.
3. Owen, J.S., Bruckdorfer, K.R., Day, R.C., and MacIntyre, N. (1982) *J. Lipid Res. 23,* 124-132.
4. Johnson, S.B., Gordon, E., McClain, C., Low, G., and Holman, R.T. (1985) *Proc. Natl. Acad. Sci. USA 82,* 1815-1818.
5. Lefkowith, J.B., Flippo, V., Sprecher, H., and Needleman, P. (1985) *J. Biol. Chem. 260,* 15736-15744.
6. Trotter, J., Flesh, I., Schmidt, B., and Ferber, E. (1982) *J. Biol. Chem. 257,* 1816-1823.
7. Flesh, I., Ecker, B., and Ferber, E. (1984) *Eur. J. Biochem. 139,* 431-437.
8. Bachelet, M., Masliah, J., Vargaftig, B.B., Béréziat, G., and Colard, O. (1986) *Biochim. Biophys. Acta 878,* 177-183.
9. Kramer, R.M., and Deykin, D. (1983) *J. Biol. Chem. 258,* 13806-13811.
10. Colard, O., Breton, M., and Béréziat, G. (1984) *Biochem. J. 222,* 657-662.
11. Aeberhard, E.E., Corbo, L., and Menkes, J.H. (1978) *Lipids 13,* 758-767.
12. Chambaz, J., Guillouzo, A., Cardot, P., Pépin, D., and Béréziat, G. (1986) *Biochim. Biophys. Acta 878,* 310-319.
13. Guguen-Guillouzo, C., Clément, B., Baffet, B. Beaumont, C., Morel-Chagny, E., Glaise, D., and Guillouzo, A. (1983) *Exp. Cell. Res. 143,* 47-54.
14. Guguen, C., Guillouzo, A., Boisnard, M., Le Cam, A., and Bourel, M. (1975) *Biol. Gastroenterol. 8,* 223-231.
15. Williams, G.M., Weisburger, E.K., and Weisburger, J.H. (1971) *Exp. Cell. Res. 69,* 106-112.
16. Chen, R.F. (1967) *J. Biol. Chem. 242,* 173-181.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
18. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. 37,* 912-917.
19. Chen, R.F. (1956) *Anal. Chem. 28,* 1756-1758.
20. Van Wijngaarden, D. (1967) *Anal. Chem. 39,* 848-849.
21. Weiner, T.W., and Sprecher, H. (1984) *Biochim. Biophys. Acta 792,* 293-303.
22. Hyman, B.T., Stoll, L.L., and Spector, A.A. (1981) *J. Biol. Chem. 256,* 8863-8866.

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Absorption and Transport of Deuterium-Substituted 2*R*,4′*R*,8′*R*-α-Tocopherol in Human Lipoproteins

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Oral administration of a single dose of tri- or hexadeuterium substituted 2*R*,4′*R*,8′*R*-α-tocopheryl acetate (d_3- or d_6-α-T-Ac) to humans was used to follow the absorption and transport of vitamin E in plasma lipoproteins. Three hr after oral administration of d_3-α-T-Ac (15 mg) to 2 subjects, plasma levels of d_3-α-T were detectable; these increased up to 10 hr, reached a plateau at 24 hr, then decreased. Following administration of d_6-α-T-Ac (15–16 mg) to 2 subjects, the percentage of deuterated tocopherol relative to the total tocopherol in chylomicrons increased more rapidly than the corresponding percentage in whole plasma. Chylomicrons and plasma lipoproteins were isolated from 2 additional subjects following administration of d_3-α-T-Ac (140 or 60 mg). The percentage of deuterated tocopherol relative to the total tocopherol increased most rapidly in chylomicrons, then in very low density lipoproteins (VLDL), followed by essentially identical increases in low and high density lipoproteins (LDL and HDL, respectively) and lastly, in the red blood cells. This pattern of appearance of deuterated tocopherol is consistent with the concept that newly absorbed vitamin E is secreted by the intestine into chylomicrons; subsequently, chylomicron remnants are taken up by the liver from which the vitamin E is secreted in VLDL. The metabolism of VLDL in the circulation results in the simultaneous delivery of vitamin E into LDL and HDL. ***Lipids 23,*** **791–797 (1988).**

Vitamin E is a fat-soluble vitamin which is transported in human plasma entirely within lipoproteins; no other transport proteins in plasma have been demonstrated (1,2). We have suggested that the transport of vitamin E and its delivery to tissues appears to be related to lipid and lipoprotein metabolism (3). Previously, two mechanisms which deliver lipids to tissues also have been recognized as delivering tocopherol to tissues. Lipoprotein lipase, which hydrolyzes triglycerides contained in lipid-rich lipoproteins (chylomicrons and very low density lipoproteins [VLDL]), also delivers tocopherol to cells, as has been demonstrated in vitro (4). The low density lipoprotein (LDL) receptor, which delivers cholesterol-rich LDL to cells, also delivers tocopherol to cells (5). Although the mechanisms for delivery of tocopherol can be studied in vitro, the question of how and where tocopherol is incorporated into lipoproteins previously was not resolvable due to the lack of an adequate metabolic tracer that could be used in vivo in humans.

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Abbreviations: d_0-α-T, nondeuterated α-tocopherol; d_3-α-T, 2*R*,4′*R*,8′*R*-α-(5-C^2H_3)tocopherol; d_6-α-T, 2*R*,4′*R*,8′*R*-α-(5,7-$[C^2H_3]_2$)-tocopherol; d_9-α-T, ambo-α-(5,7,8-$[C^2H_3]_3$)tocopherol; d_3-α-T-Ac, d_3-α-T acetate; d_6-α-T-Ac, d_6-α-T acetate; EDTA, ethylenediaminetetraacetic acid; GC-MS, gas chromatography-mass spectrometry; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; RBC, red blood cells; VLDL, very low density lipoprotein.

Recently, we have developed a protocol for the synthesis of 2*R*,4′*R*,8′*R*-α-tocopherol (the "natural" stereoisomer) substituted with a precise amount of deuterium and have used deuterated tocopherol to measure the in vivo accumulation of vitamin E in a variety of rat tissues (6). Because deuterium is a stable isotope of hydrogen and presents no hazard to health, deuterium-substituted vitamin E can be safely administered to humans. While many studies have been carried out on vitamin E absorption using single doses of vitamin E (natural and synthetic) or of radioactively labeled vitamin E, the use of deuterium-substituted vitamin E is new and has certain advantages. These include: lack of radioactive toxicity, excellent quantitation at low doses and long-term stability after preparation.

This report describes the use of deuterated α-tocopherol in humans for the study of the absorption of vitamin E and its transport in plasma lipoproteins.

METHODS

Deuterated tocopherol. The procedure for the synthesis of deuterated tocopherol has been described previously (6,7). 2*R*,4′*R*,8′*R*-α-Tocopheryl acetate substituted with either three deuterium atoms (2*R*,4′*R*,8′*R*-α-(5-C^2H_3)tocopheryl acetate; d_3-α-T-Ac) or six deuterium atoms (2*R*,4′*R*,8′*R*-α-(5,7-$[C^2H_3]_2$)tocopheryl acetate; d_6-α-T-Ac) was administered to subjects. The α-tocopherol content of these compounds was shown to be >99% both by thin layer chromatography (TLC) and by gas chromatography-mass spectrometry (GC-MS). The degree of incorporation of the nominal amount of deuterium into each compound was determined precisely by GC-MS and was found to be greater than 85%. All data were adjusted to reflect the true level of the deuterium-substituted tocopherol in the blood samples. The deuterated tocopheryl acetate was taken as a single dose of a weighed quantity of the pure material in a hard gelatin slip-joint capsule.

Subjects. The subjects studied were normal human volunteers without metabolic abnormalities of lipid metabolism and were of normal weight. The subjects gave informed consent and the study was carried out within the guidelines established by the Institutional Review Board of New York University School of Medicine. Following an overnight fast (12 hr), blood was drawn for a pretreatment sample. The subject then swallowed a capsule containing a known weight of deuterated α-tocopheryl acetate, as indicated in the text, consumed a usual breakfast immediately afterwards and followed the usual pattern of daily activities and meals. Blood was drawn into vacutainer tubes containing 0.05 ml 15% ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson, Rutherford, NJ) at the indicated time intervals following the capsule administration. Plasma was immediately separated (within 5 min of blood drawing) from red cells by centrifugation at 20 C. The red cells were washed as previously described (8,9), by repeated resuspension in

phosphate buffered saline and centrifugation at 20 C. Aliquots of known hematocrit were stored at −70 C until shipment on dry ice to Ottawa, Canada, for analysis.

Isolation of lipoproteins by ultracentrifugation. Chylomicrons and lipoproteins were isolated using the newly available TL100 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). Chylomicrons were isolated by a modification of the technique described by Weintraub et al. (10). Plasma (1 ml) was overlayered with 1 ml dialysis buffer (0.15 M NaCl, 0.3 mM EDTA, pH 7.4) in polyallomer centrifuge tubes (11 × 34 mm) and centrifuged with a TLS55 rotor for 8 min at 40,000 rpm in the TL100 ultracentrifuge at 20 C using acceleration and deceleration factors of 3. The chylomicrons were recovered from the top of the tube by tube slicing with a Centritube Slicer (Beckman Instruments, Inc.). The bottom fraction then was aliquoted (1 ml) in duplicate into polycarbonate tubes (11 × 34 mm; Beckman Instruments, Inc.) for isolation of plasma lipoproteins as described (11). The VLDL fraction ($d < 1.006$) was isolated by centrifuging the samples in a TLA-100.2 fixed angle rotor (Beckman Instruments, Inc.) at 435,680 × g for 2 hr at 8 C. The upper 0.25 ml was collected by slicing the tubes. The bottom fraction was collected and the density adjusted to 1.063 g/ml with solid KBr. The samples were then centrifuged at 435,680 × g for 2 hr at 8 C and the top ($1.006 < d < 1.063$, LDL) and bottom ($d > 1.063$, HDL) fractions were recovered as described above. Upon isolation, the lipoprotein fractions were frozen and stored at −70 C until overnight shipment to Ottawa, Canada, on dry ice. The KBr was not removed from the samples prior to analysis.

Analysis of deuterated tocopherol. The relative amounts of deuterated α-tocopherol were determined as previously described (6). In addition, ambo-α-(5,7,8-$[C^2H]_3$)tocopherol (d_9-α-T) was synthesized and was used as an internal standard in order to quantitate the absolute amounts of nondeuterated and d_3- or d_6-substituted α-substituted α-tocopherols. The d_9-α-T, dissolved in *n*-decane, was added to the samples prior to extraction. The d_3-α-T measurements were corrected for the contribution of natural abundance isotopes present in the nondeuterated α-tocopherol. This correction is negligible for d_6-α-T, which permits more precise measurements of very low levels of deuterated tocopherol. This would make d_6-α-T the compound of choice in these experiments. However, d_6-α-T is more expensive to prepare than d_3-α-T and furthermore, pure δ-tocopherol, the d_6-precursor, is more difficult to obtain than pure γ-tocopherol, the d_3-precursor.

RESULTS

Following the oral administration of d_3-α-T-Ac (15 mg) to two male subjects, the percentage of d_3-α-tocopherol (d_3-α-T) relative to the total α-tocopherol present in plasma and red cells was determined. As shown in Table 1, the percentage of d_3-α-T increased during the first 24 hr, reaching nearly 8% of the total tocopherol present in the plasma of subject 2. As would be expected from the known rapid exchange of α-tocopherol between plasma and red cells (8,12,13), the red cells also contained d_3-α-T, with the fractional increase in the deuterated α-tocopherol following a pattern similar to that of the plasma (Table 1).

TABLE 1

Appearance of d_3-α-T in Plasma and Red Cells of Two Male Subjects Following Oral Administration of d_3-α-T-Ac (15 mg)

Subject	Time (hr)	% d_3-α-T[a] Plasma	% d_3-α-T[a] Red cells
1	0	0	0
	5	0.4	0.6
	10	3.1	1.6
	27	4.1	4.6
	51	3.3	4.9
2	0	0	0
	4	0.4	0.4
	9	7.6	3.2
	26	7.0	7.2
	50	5.1	5.8

[a]Percentage of d_3-α-T relative to the total α-tocopherol, $100 \times [d_3\text{-}\alpha\text{-T}/(d_3\text{-}\alpha\text{-T} + d_0\text{-}\alpha\text{-T})]$.

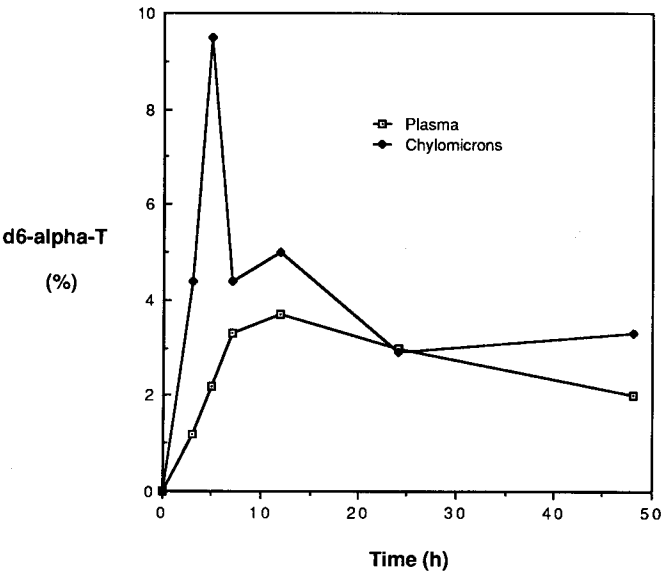

FIG. 1. Percentages of d_6-α-T of the total tocopherol present in the chylomicron fraction and in the plasma of subject 3 (female) at the indicated intervals following oral administration of d_6-α-T-Ac (16 mg).

To assess the mechanism of vitamin E transport from the intestine, two subjects (one male, one female) were given d_6-α-T-Ac (15–16 mg) and plasma was collected at intervals for 48 hr. The percentage of d_6-α-T relative to the total α-tocopherol content of both the chylomicron fraction and whole plasma were determined. As shown in Figure 1, the d_6-α-T in the chylomicron fraction of the plasma of the female subject (subject 3) had increased after 5 hr to nearly 10% of the total α-tocopherol in this lipoprotein fraction. This increase in the percentage of d_6-α-T occurred more rapidly in the chylomicron fraction than the corresponding increase in the plasma, and the maximum percentage was greater in the chylomicrons than in the plasma. A second peak in the chylomicron fraction was observed at 12 hr. Qualitatively similar data were obtained for the male subject (subject 4, data not shown).

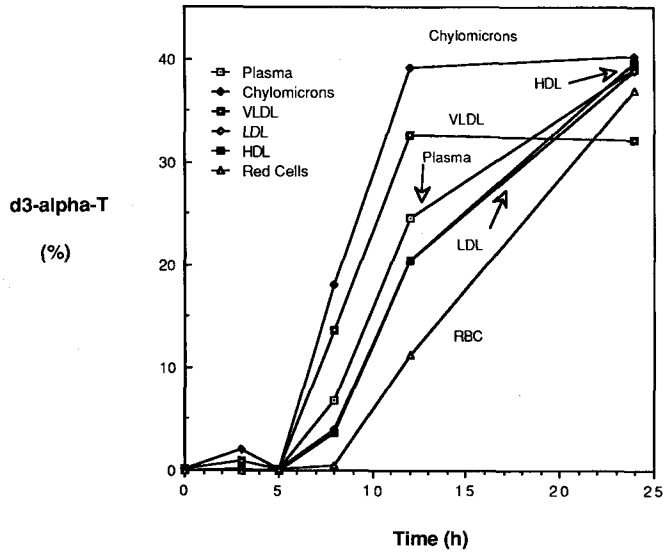

FIG. 2. Percentages of d_3-α-T in chylomicrons, VLDL, LDL, HDL, plasma and RBC relative to the total α-T in each fraction of subject 5 at the indicated intervals following oral administration of d_3-α-T-AC (140 mg).

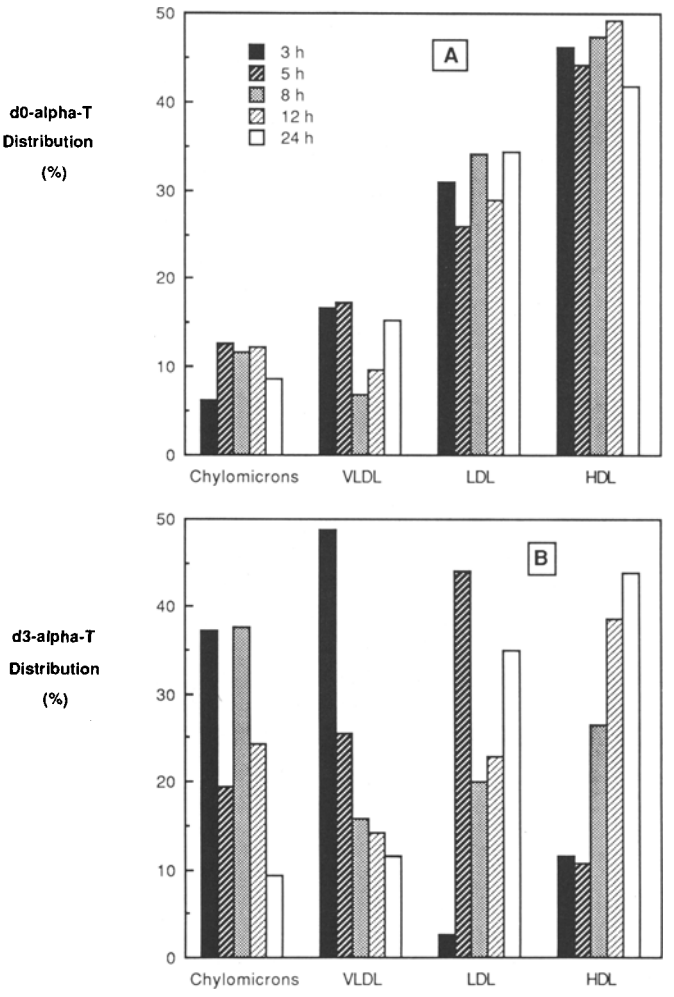

FIG. 3. Distribution (%) of nondeuterated (d_0-α-T, A) and deuterated (d_3-α-T, B) tocopherol between chylomicrons, VLDL, LDL and HDL isolated from the plasma of subject 5 at each of the time points following oral administration of d_3-α-T-Ac (140 mg).

The purpose of this study was to follow the incorporation of vitamin E into all of the plasma lipoproteins. Therefore, a larger dose (140 mg) of d_3-α-T-Ac was administered to subject 5 and the distribution of d_3-α-T in the major lipoprotein fractions was assessed after various intervals of time. As shown in Figure 2, the chylomicron fraction contained the largest percentage of d_3-α-T at all of the time points measured. Unlike the data shown in Figure 1, the deuterated tocopherol reached a maximum in the chylomicron fraction at 12 hr rather than at 5 hr. The lipoprotein fraction that showed the second fastest increase in the percentage content of d_3-α-T was the VLDL fraction, a result which is consistent with uptake of chylomicron remnants by the liver and secretion of the newly absorbed lipids in VLDL particles. The percentages of d_3-α-T in the LDL and HDL fractions were virtually identical at all times, with the highest percentage of d_3-α-T observed at 24 hr.

The absolute quantities of nondeuterated α-tocopherol (d_0-α-T) and of d_3-α-T measured in the plasma, in the four lipoprotein fractions (chylomicrons, VLDL, LDL and HDL), and in the red blood cells of subject 5 are given for each time point in Table 2. The sums of the quantities of d_0-α-T and of d_3-α-T recovered from all of the lipoprotein fraction were between 80–110% of the values in whole plasma. Figure 3 shows the percentages of d_0-α-T and of d_3-α-T in each lipoprotein fraction based on the sum of the quantities of each of these materials recovered at the corresponding time point. From the distribution of d_0-α-T in each lipoprotein fraction (Fig. 3A), it is apparent that most (40–50%) of the tocopherol is present in the HDL fraction of this subject, while the chylomicron and VLDL fractions each contain less than 17%. In marked contrast, Figure 3B shows that at 3 hr the chylomicron fraction contained nearly 40% of the d_3-α-T present in the plasma, with the VLDL fraction containing almost 50%. At 5 hr the d_3-α-T in the chylomicron fraction decreased to 19% with a corresponding increase to 44% in LDL. The remaining time points (8–24 hr) show the stepwise decrease in the d_3-α-T present in the triglyceride-rich particles with the concomitant increases in d_3-α-T in the LDL and HDL particles, finally resulting by 24 hr in the similar distribution of d_3-α-T and d_0-α-T.

Subject 6 was given a smaller amount (60 mg) of d_3-α-T-Ac than subject 5 because the 140-mg dose given to subject 5 produced a plateau in the d_3-α-T/d_0-α-T ratio in the chylomicron fraction, suggesting that the intestine continued to secrete chylomicrons containing d_3-α-T for as long as 24 hr after administration of the dose. As can be seen from Figure 4, a similar pattern of increases in the percentage of d_3-α-T in the lipoprotein fractions was observed in subject 6 as in subject 5 (Fig. 2). However, in subject 6, the percentage of d_3-α-T in the VLDL fraction increased at 8 hr and remained above that seen in the chylomicron fraction. The absolute quantities of d_0-α-T and of d_3-α-T in the plasma, lipoprotein fractions and red blood cells of subject 6 are given in Table 3 for each time point. Table 3 also includes the total quantities of

TABLE 2

Absolute and Percent Relative Concentrations of d_0- and d_3-α-Tocopherol in Plasma, Lipoprotein and Red Blood Cell Fractions of Subject 5

Blood fraction	Time (hr)	d_0-α-T[a]	d_3-α-T[a]	d_3-α-T %[b]	$\frac{d_0\text{-}\alpha\text{-T}^c}{d_0\text{-}\alpha\text{-T(sum)}}$ %	$\frac{d_3\text{-}\alpha\text{-T}^c}{d_3\text{-}\alpha\text{-T(sum)}}$ %
Plasma	0	39.0	0.00	0.0		
	3	38.8	0.00	0.0		
	5	39.3	0.03	0.1		
	8	37.0	2.70	6.8		
	12	35.7	11.6	24.5		
	24	29.8	19.0	38.9		
	48	33.2	15.3	31.6		
Chylomicrons	0	5.78	0.01	0.1		
	3	2.18	0.05	2.1	6.2	37.2
	5	4.53	0.01	0.2	12.6	19.5
	8	3.72	0.82	18.0	11.7	37.7
	12	4.25	2.73	39.1	12.3	24.2
	24	2.82	1.90	40.3	8.6	9.4
VLDL	0	4.61	0.00	0.1		
	3	5.87	0.06	1.0	16.7	48.7
	5	6.20	0.01	0.2	17.3	25.5
	8	2.19	0.34	13.6	6.9	15.8
	12	3.30	1.60	32.7	9.6	14.2
	24	4.99	2.35	32.1	15.3	11.6
LDL	0	n.d.	n.d.			
	3	10.8	0.00	0.0	31.0	2.6
	5	9.3	0.02	0.2	25.9	44.2
	8	10.9	0.44	3.9	34.1	20.0
	12	9.9	2.57	20.5	28.9	22.8
	24	11.2	7.12	38.8	34.4	35.0
HDL	0	n.d.	n.d.			
	3	16.1	0.01	0.1	46.1	11.6
	5	15.8	0.00	0.0	44.1	10.8
	8	15.1	0.58	3.7	47.3	26.5
	12	17.0	4.36	20.5	49.2	38.7
	24	13.6	8.95	39.6	41.7	44.0
RBC	0	5.05	0.00	0.0		
	3	6.01	0.00	0.0		
	5	3.56	0.01	0.2		
	8	3.39	0.02	0.5		
	12	2.89	0.36	11.2		
	24	2.90	1.70	36.9		
	48	3.56	1.93	35.2		

[a]Concentrations are in nmol/ml of plasma or packed red cells.

[b]Percentage d_3-α-T relative to total α-T in each fraction (i.e., d_3-α-T + d_0-α-T).

[c]Percentage relative to sum of chylomicron, VLDL, LDL and HDL fractions at each time point.

n.d., not determined.

α-tocopherol and the percentages of d_0-α-T and d_3-α-T in each lipoprotein fraction (based on the sum of d_0-α-T and d_3-α-T, respectively, in these four fractions). The sums of the d_0-α-T recovered from each lipoprotein fraction were 49–108% of the corresponding quantities measured in whole plasma.

As shown in Figure 5A, the distribution of d_0-α-T in the plasma of subject 6 was equally divided into LDL and HDL, with little in the triglyceride-rich lipoproteins. At times 3.5 and 5 hr, the percentage of d_0-α-T in the chylomicron fraction decreased in response to the absorption of d_3-α-T, although only 8% and 10% of the total d_3-α-T was present in chylomicrons (Figure 5B). It is likely that this subject had a peak in chylomicron d_3-α-T prior to the 3.5 hr time point, as the HDL fraction by this time contained 55% of the plasma d_3-α-T. This subject appears to rapidly catabolize triglyceride-rich particles and by 8 hr, d_3-α-T is distributed identically to d_0-α-T.

DISCUSSION

Deuterium-substituted α-tocopherol was used in this study to follow vitamin E transport in human plasma. The technique allows the α-tocopherol derived from newly-ingested α-tocopheryl acetate to be distinguished from existing, nondeuterated α-tocopherol present in the individual. We have demonstrated that a dose of 15 mg of deuterated α-tocopheryl acetate gives measurable

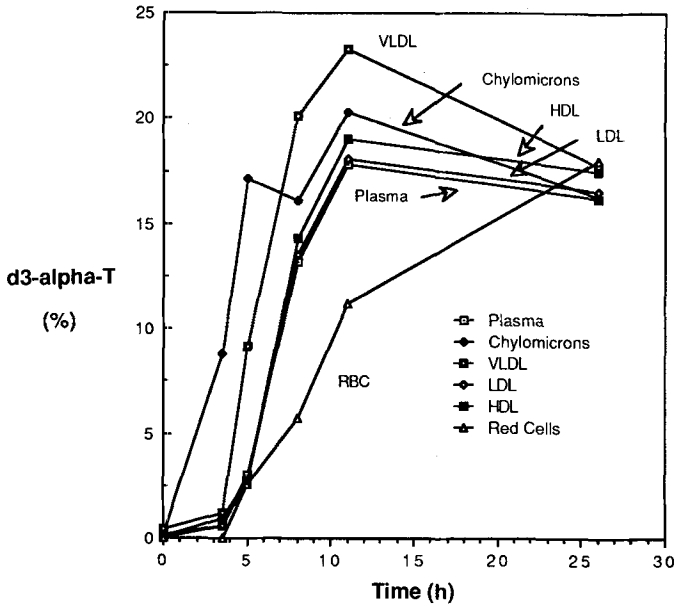

FIG. 4. Percentages of d_3-α-T in each of the lipoprotein fractions, plasma and RBC relative to the total α-T in each fraction of subject 6 at the indicated intervals following oral administration of d_3-α-T-Ac (60 mg).

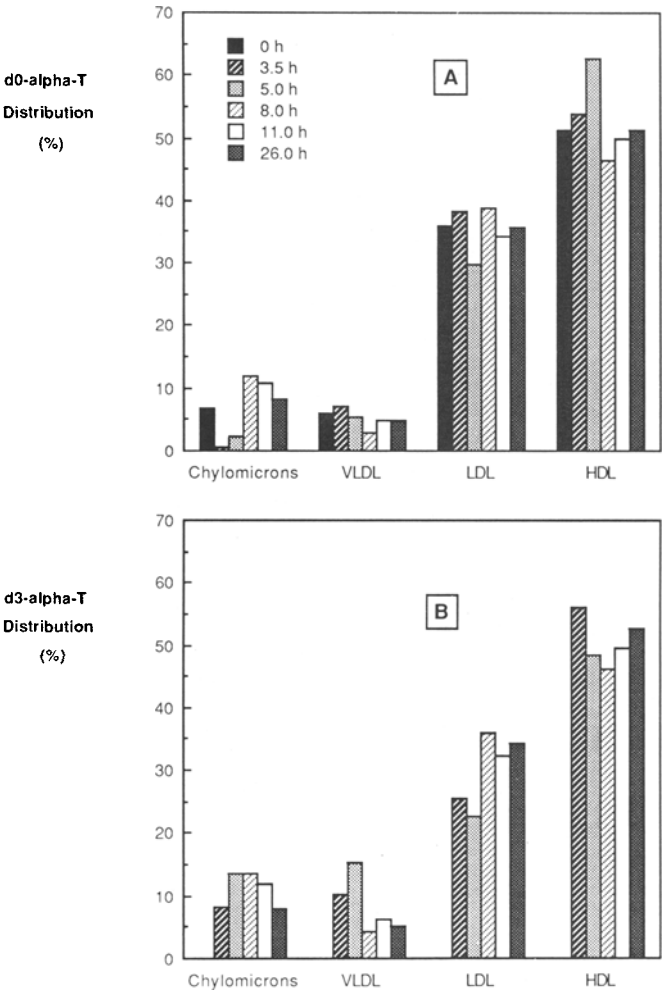

FIG. 5. Distribution (%) of nondeuterated (d_0-α-T, A) and deuterated (d_3-α-T, B) tocopherol within each of the lipoprotein fractions isolated from the plasma of subject 6 at the indicated intervals following the oral administration of d_3-α-T-Ac (60 mg).

quantities of deuterated α-tocopherol in the plasma and is sufficient to document its incorporation into chylomicrons. The time course of the transport of α-tocopherol in lipoproteins was studied using 140-mg and 60-mg doses of d_3-α-T-Ac. It can be seen from the data in Tables 2 and 3, that the effect of these fairly large doses of d_3-α-T is to produce rather small but clearly discernible increases in the total quantity of vitamin E (i.e., d_0-α-T + d_3-α-T) in the plasma of subjects 5 and 6, in most of the lipoprotein fractions of subject 5 and in the red blood cells.

α-Tocopherol from orally-administered deuterated α-tocopheryl acetate appeared first in the chylomicron fraction of the plasma. The percentage of deuterated tocopherol increased in this fraction before increasing in the whole plasma and before the increase in any of the other lipoprotein fractions (Figs. 2 and 4). The VLDL fraction was the next to become labeled. This result is consistent with the secretion of chylomicrons by the intestine, uptake of chylomicron remnants by the liver and secretion by the liver of α-tocopherol in newly synthesized VLDL. Both chylomicrons and VLDL are substrates for lipoprotein lipase, which hydrolyzes the triglycerides present in these lipid-rich particles, producing chylomicron remnants and intermediate density lipoproteins (IDL), respectively. Traber et al. (4) have shown in vitro that lipoprotein lipase transfers tocopherol to cells during the hydrolysis of triglyceride-rich particles. Thus, the newly absorbed vitamin E is first incorporated into chylomicrons, then, during the production of chylomicron remnants by lipoprotein lipase (14), some α-tocopherol is transferred to tissues, but much of the vitamin E remains in the remnants which are subsequently taken up by the liver. The liver then secretes the newly absorbed tocopherol in VLDL particles, which also are subject to catabolism by lipoprotein lipase. During the latter process, some IDL particles are converted to LDL, while some are taken up by the liver (14).

From the pattern of deuterated tocopherol present in the LDL and HDL fractions (Figs. 2 and 4), it is clear that the increase in the percentage of d_3-α-T in these two fractions occurs after the increase of deuterated tocopherol in VLDL. The striking finding shown in Figures 3 and 5 is the demonstration of the differences in tocopherol transport in the two subjects, despite the apparent similarities of Figures 2 and 4. In subject 5 (Fig. 3), the d_3-α-T was predominantly in the triglyceride-rich lipoproteins at early time points, with little d_3-α-T in the HDL fraction until the 12- and 24-hr time points. By contrast, in subject 6, 56% of the d_3-α-T tocopherol present in the plasma at 3.5 hr was in the HDL fraction—the chylomicron fraction never contained a major portion of the plasma d_3-α-T. The differences in the two subjects are probably a result of the larger dose of d_3-α-T-Ac given to subject 5, and to differences in the lipoprotein metabolism in the two subjects. The results do not unequivocally demonstrate whether the majority of tocopherol in the plasma arises from the catabolism of VLDL to LDL, as the data in Figure 3B suggest, or whether the majority

TABLE 3

Absolute and Percent Relative Concentrations of d_0- and d_3-α-Tocopherol in Plasma, Lipoprotein and Red Blood Cell Fractions of Subject 6

Blood fraction	Time (hr)	d_0-α-T[a]	d_3-α-T[a]	d_3-α-T %[b]	$\frac{d_0\text{-}\alpha\text{-}T^c}{d_0\text{-}\alpha\text{-}T(sum)}$ (%)	$\frac{d_3\text{-}\alpha\text{-}T^c}{d_3\text{-}\alpha\text{-}T(sum)}$ (%)
Plasma	0.0	27.1	0.00	0.0		
	3.5	26.3	0.15	0.6		
	5.0	25.4	0.79	3.0		
	8.0	24.9	3.78	13.2		
	11.0	26.1	5.65	17.8		
	26.0	25.4	4.92	16.2		
	53.0	19.9	2.22	10.0		
Chylomicrons	0.0	1.53	0.00	0.2	6.7	—
	3.5	0.13	0.01	8.8	0.7	8.1
	5.0	0.29	0.06	17.1	2.4	13.6
	8.0	1.71	0.33	16.1	11.9	13.6
	11.0	1.54	0.39	20.3	10.9	11.8
	26.0	1.34	0.26	16.3	8.3	7.9
VLDL	0.0	1.34	0.01	0.4	5.9	—
	3.5	1.30	0.02	1.2	7.1	10.3
	5.0	0.67	0.07	9.2	5.4	15.2
	8.0	0.40	0.10	20.1	2.8	4.2
	11.0	0.68	0.21	23.3	4.8	6.2
	26.0	0.77	0.17	17.7	4.8	5.0
LDL	0.0	8.20	0.01	0.2	36.1	—
	3.5	7.05	0.04	0.6	38.3	25.4
	5.0	3.69	0.10	2.7	29.7	22.7
	8.0	5.55	0.87	13.5	38.7	36.1
	11.0	4.81	1.06	18.1	34.3	32.2
	26.0	5.74	1.14	16.5	35.7	34.4
HDL	0.0	11.7	0.01	0.1	51.3	—
	3.5	9.90	0.09	0.9	53.9	56.2
	5.0	7.76	0.21	2.7	62.5	48.4
	8.0	6.67	1.11	14.3	46.6	46.2
	11.0	7.00	1.64	19.0	49.9	49.7
	26.0	8.24	1.75	17.5	51.2	52.7
RBC	0.0	n.d.	n.d.	—		
	3.5	1.14	0.00	0.0		
	5.0	0.58	0.02	2.6		
	8.0	2.09	0.13	5.7		
	11.0	2.08	0.26	11.2		
	26.0	0.85	0.19	18.0		
	53.0	0.58	0.07	11.1		

[a]Concentrations are in nmol/ml of plasma or packed red cells.

[b]Percentage d_3-α-T relative to the total α-T present in each fraction (i.e., d_3-α-T + d_0-α-T).

[c]Percentage relative to sum of chylomicron, VLDL, LDL and HDL fractions at each time point.

n.d., not determined.

of plasma tocopherol arises from the transfer of tocopherol with surface components to HDL (14) during the hydrolysis of triglyceride-rich particles, as Figure 5B suggests. The ability of tocopherol to exchange between LDL and HDL (12) quickly results in the equilibration of the newly ingested tocopherol in these two fractions, such that the percentages of d_3-α-T of the total tocopherol are similar in LDL and HDL. It appears that differences in lipid metabolism and in lipoprotein synthesis and turnover between individuals are most important in determining the distribution of tocopherol between the individual lipoproteins.

In conclusion, the data presented in this study using deuterium-labeled α-tocopherol demonstrate that vitamin E is incorporated and transported in the lipoproteins in a manner consistent with the known mechanisms of lipid and lipoprotein transport and catabolism. The use of deuterium-substituted α-tocopherol will be invaluable for assessing the bioavailability of different stereoisomers of α-tocopherol, for measuring the absorption and transport of vitamin E in patients at risk from vitamin E deficiency due to abetalipoproteinemia, cholestatic liver disease, cystic fibrosis and other related diseases.

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REFERENCES

1. Bjornson, L.K., Kayden, H.J., Miller, E., and Moshell, A.N. (1976) *J. Lipid Res. 17,* 343–352.
2. Behrens, W.A., Thompson, J.N., and Madere, R. (1982) *Am. J. Clin. Nutr. 35,* 691–696.
3. Kayden, H.J., and Traber, M.G. (1987) in *Clinical and Nutritional Aspects of Vitamin E* (Hayaishi, O., and Mino, M., eds.) pp. 129–138, Elsevier Science Publishers B.V., Amsterdam, Netherlands.
4. Traber, M.G., Olivercrona T., and Kayden, H.J. (1985) *J. Clin. Invest. 75,* 1729–1734.
5. Traber, M.G., and Kayden, H.J. (1984) *Am. J. Clin. Nutr. 40,* 747–751.
6. Ingold, K.U., Burton, G.W., Foster, D.O., Hughes, L., Lindsay, D.A., and Webb, A. (1987) *Lipids 22,* 163–172.
7. Ingold, K.U., Hughes, L., Slaby, M., and Burton, G.W. (1987) *J. Labelled Compds. Radiopharm. 24,* 817–831.
8. Cheng, S.C., Burton, G.W., Ingold, K.U., and Foster, D.O. (1987) *Lipids 22,* 469–473.
9. Hatam, L.J., and Kayden, H.J. (1979) *J. Lipid Res. 20,* 639–645.
10. Weintraub, M.S., Eisenberg, S., and Breslow, J.L. (1987) *J. Clin. Invest. 79,* 1110–1119.
11. Traber, M.G., Kayden, H.J., and Rindler, M. (1987) *J. Lipid Res. 28,* 1350–63.
12. Kayden, H.J., and Bjornson, L.K. (1972) *Ann. N.Y. Acad. Sci. 203,* 127–140.
13. Bjornson, L.K., Gniewkowski, C., and Kayden, H.J. (1975) *J. Lipid Res. 16,* 39–53.
14. Gotto, A.M., Pownall, H.J., and Havel, R.J. (1986) *Methods Enzym. 128,* 3–41.

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Effect of Previous Nutritional Status on the Formation of Cholesterol Gallstones in the Prairie Dog

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In the prairie dog model of cholesterol cholelithiasis, a high incidence of gallstones is achieved by feeding a semipurified lithogenic diet containing 0.4% cholesterol for 2 mo. On occasion, we noted a decrease in the percentage of animals with gallstones from 90–100% to 50–55%. To explain this phenomenon, we studied the effect of dietary history on gallstone formation. After weaning, animals were fed either rodent chow or alfalfa plus corn (mo 0–3) followed by a cross-over experiment at mo 4–6. Gallstone formation then was studied by feeding the lithogenic diet from mo 7 to 8. At sacrifice, the incidences of gallstones, biliary lipids and tissue cholesterol levels were correlated with dietary history. The incidence of gallstones was 100% only in animals fed the alfalfa-corn diet from weaning to 3 mo. In addition, the feeding of the alfalfa-corn diet at mo 4–6 increased gallstone incidence from 65% to 86%. The lithogenic index of all groups was highest when the animals received only alfalfa-corn prior to the lithogenic stimulus. The activity of hepatic HMG-CoA reductase was elevated in animals fed alfalfa-corn from weaning to 8 mo, suggesting that this diet stimulates hepatic cholesterol synthesis, leading to increased biliary cholesterol secretion. It is concluded that previous nutritional conditioning affects the incidence of gallstones. The prairie dog is a useful model of cholesterol cholelithiasis, but the dietary history of the animals plays an important role in lithogenesis.
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The formation and prevention of cholesterol gallstones have been studied in a number of animal models, most frequently in the mouse (1), hamster (2–6) and prairie dog (7–12). Studies in man have been concerned largely with dissolution of stones (13), but the prevention of stone recurrence following dissolution by chenodeoxycholic acid, ursodeoxycholic acid, methyl tertiary butyl ether or physical means has recently become of considerable interest (14). Dissolution of cholesterol gallstones in man following the oral administration of chenodeoxycholic acid (750 mg per day) was the objective of extensive clinical trials in the United States (13) and Canada (15), but the results were disappointing, apparently because the dose of the drug per unit of body wt was too low. Recently, considerably higher dissolution rates were obtained by European investigators using dosages of 12–16 mg/kg/day (16). Nevertheless, there exists still the need for developing a second generation of cholelitholytic drugs that possess greater efficacy than chenodeoxycholic acid and ursodeoxycholic acid and do not give rise to the potentially hepatoxic lithocholic acid via bacterial 7-dehydroxylation.

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Abbreviations: GLC, gas liquid chromatography; GLC-MS, gas liquid chromatography-mass spectrometry.

Our laboratory has been moderately successful in studying the prevention of gallstones by hydrophobic and hydrophilic bile acids in the prairie dog (10,11). For this purpose, gallstones were induced routinely in 90–100% of the animals by the administration of a semipurified diet containing 0.4% cholesterol. Occasionally, for reasons not entirely clear to us, the incidence of gallstones in animals fed the lithogenic diet for the standard period of 8 wk decreased to less than 50%. We decided to investigate this undesirable phenomenon, because a low incidence of gallstones in the control groups would make the evaluation of gallstone-preventing or dissolving agents very difficult.

This manuscript describes the effect of the dietary history of the prairie dogs on the subsequent incidence of gallstones after animals are placed on the semipurified, lithogenic diet. The data strongly suggest that the dietary history of the prairie dogs plays a role in cholesterol metabolism and gallstone formation.

MATERIALS AND METHODS

Animals and diets. Male prairie dogs *(Cynomys ludovicianus)* were bred in captivity by R-Zoo, Neshboro, WI. The animals were maintained at the R-Zoo facility from weaning to 5.5 mo. They were received at the Beth Israel Medical Center Animal Facility and maintained from 5.5 mo to age 8 mo, when the experiment was completed. The animals were fed either Purina rodent chow or alfalfa and whole grain corn (50:50, w/w), ad libitum, from weaning to 6 mo of age as summarized in Table 1. For periods 1 and 2, these diets were fed at R-Zoo (0–5.5 mo) or Beth Israel (5.5–6 mo). The animals were quarantined for the last 2 wk of mo 6. At the start of period 3 (mo 7), the animals were weighed and then fed the following: (a) semipurified diet plus 0.4% cholesterol (lithogenic diet—fed to groups 2, 3, 5 and 6), (b) chow (group 1) or (c) alfalfa-corn (group 4). The lithogenic diet was prepared by Teklad (Madison, WI) and consisted of sucrose, 51.9%; corn starch, 13.9%; soy protein, 21.0%; corn oil, 1.6%; cellulose, 2.6%; vitamin mix, 1.0% (Teklad 40860); and mineral mix, 4.0% (Teklad 40060). Cholesterol (0.4%) was incorporated as egg yolk powder (4%). Period 3 lasted 8 wk; food intake was limited to 40 g/day with water supplied ad libitum. The animals were kept on an alternating 12-hr light–12-hr dark cycle. Food was removed during the 24 hr prior to sacrifice of the animals (mo 8) to insure adequate quantities of bile in the gallbladder for biliary lipid analyses. The animals were anesthetized with 100 mg of ketamine hydrochloride (Bristol Labs, Syracuse, NY) and 20 mg of xylazine (Haver Lockhart, Shawnee, KA). They were killed by exsanguination and blood was collected for determination of serum cholesterol. The gallbladder was removed and bile was aspirated with a Hamilton syringe. The fresh bile was immediately examined by polarized light microscopy (Olympus MCHAP microscope, Olympus Corp., Lake Success, NY) to determine the presence of cholesterol crystals, liquid crystals and cholesterol

gallstones (17). The liver was excised and weighed and aliquots removed for cholesterol determination and preparation of microsomes.

Biliary lipid composition. Gallbladder bile obtained at sacrifice was immediately centrifuged at 2000 × *g* for 10 min. Aliquots (50 μl) were prepared and used for determination of the three biliary lipids (10,11). The lithogenic indices were calculated using the methods of Carey (18) and Kuroki et al. (19).

Enzyme assays. Liver microsomes were prepared from samples of fresh liver. HMG-CoA reductase and cholesterol 7α-hydroxylase were measured using methods described earlier (10,11).

Gas liquid chromatography and gas liquid chromatography-mass spectrometry. Gas liquid chromatography (GLC) of cholesterol in liver, plasma and bile was carried out as described earlier (10,11). Biliary bile acids were separated and quantitated as their methyl ester acetates on a Hewlett-Packard 5831A gas chromatograph equipped with a capillary column (SPB-5, 25 m, Supelco Inc., Bellefonte, PA). The conditions for the GLC analysis were: injector temp, 270 C; detector temp, 270 C; auxiliary temp, 270 C; temp 1, 200 C for 2 min; temp 2, 270 C for 25 min (rate 10 C/min). In every group, the identity of the bile acids was confirmed by gas liquid chromatography-mass spectrometry (GLC-MS) using a Hewlett-Packard 5992B mass spectrometer. The conditions used were: 4 ft glass column, 2 mm ID, 4 mm OD, packed with 3% SP 2250 on 100/120 mesh Supelcoport; column temp, 260 C; injector temp, 265 C; source pressure, 1.5×10^{-6} torr; source temp, 140 C.

Reference compounds. 5α-Cholestane (Applied Science) was used as an internal standard for quantitation of cholesterol. 3α,7α-Dihydroxy-12-keto-5β-cholanoic acid (Steraloids, Wilton, NH) was used as a recovery standard for biliary bile acids.

Statistical calculations. All data are reported as mean ± SEM. Analysis of variance was used to determine the F statistic. Students' t-test was used for the values where the F statistic was significant (20). Chi square was used to determine the statistical significance of gallstone incidence between groups (20).

RESULTS

All prairie dogs were bred in captivity and fed the various diets after weaning to the end of the experiment (Table 1). The average initial and final weights of the animals in all groups were similar and did not differ statistically. Food intake and fecal outputs, monitored during period 3 for the six groups, also were similar.

At sacrifice, the incidence of cholesterol gallstones, cholesterol crystals, biliary lipids, activities of the rate-limiting enzymes of cholesterol and bile acid synthesis and tissue-cholesterol levels were correlated with dietary history. Incidences of gallstones and cholesterol crystals are summarized in Table 2. Animals given the chow diet during periods 1 and 2 had a lower incidence of both stones and crystals, regardless of the diet given in period 3 (groups 1 and 2 vs 4 and 5). Stone incidence was found to be greater for groups fed alfalfa-corn in period 1 (groups 4-6 vs 1-3). The highest stone incidence was found in animals fed alfalfa-corn for periods 1 and 2 followed by the lithogenic diet in period 3 (group 5 vs group 2).

The data summarized in Table 3 show levels of cholesterol in liver, plasma and bile and the activities of the rate-limiting enzymes of cholesterol and bile acid synthesis, HMG-CoA reductase and cholesterol 7α-hydroxylase. Liver cholesterol levels were elevated in animals fed the lithogenic (0.4% cholesterol) diet during period 3. Feeding either chow or alfalfa-corn during periods 1 and/or 2 did not affect liver cholesterol. However, plasma cholesterol levels, which also were elevated by feeding the lithogenic diet, were highest for animals fed only the alfalfa-corn during periods 1 and 2. Animals given only chow or alfalfa-corn during all three feeding periods had normal liver and plasma cholesterol levels. Bile cholesterol levels were generally higher with administration of the lithogenic diet (groups 2, 3, 5 and 6). The activity of HMG-CoA reductase showed several interesting differences. First, animals fed alfalfa-corn (group 4) had an enzyme activity more than 2 times that of animals fed only chow (group 1). As expected, feeding the high cholesterol lithogenic diet (groups 2, 3, 5 and 6) led to a decrease in enzyme activity (10,21). Cholesterol 7α-hydroxylase activity averaged from 8 to 17 pmoles/mg protein/min for the experimental groups.

The data summarized in Table 4 illustrate the effect of the various dietary regimens on biliary lipid composition and lithogenic index of the bile. All groups fed the lithogenic diet had increased levels of cholesterol in the bile. This was coupled with an increased level of biliary phospholipid. The total lipid concentration for all groups ranged from 7.1 to 9.2 g/dl. Animals fed the lithogenic

TABLE 1

Experimental Design of Eight-Month Feeding Experiment[a]

Group number	Number of animals	Period 1 (1-3 mo)	Period 2 (4-6 mo)	Period 3 (7-8 mo)
1	4	Chow (C)	Chow (C)	Chow (C)
2	17	Chow (C)	Chow (C)	SSD + 0.4% cholesterol (L)
3	7	Chow (C)	Alfalfa-corn (A)	SSD + 0.4% cholesterol (L)
4	4	Alfalfa-corn (A)	Alfalfa-corn (A)	Alfalfa-corn (A)
5	17	Alfalfa-corn (A)	Alfalfa-corn (A)	SSD + 0.4% cholesterol (L)
6	8	Alfalfa-corn (A)	Chow (C)	SSD + 0.4% cholesterol (L)

[a]Animals were divided into six experimental groups and fed either chow or alfalfa-corn in periods 1 and 2 followed by the lithogenic diet (groups 2, 3, 5 and 6) or the same diet (groups 1 and 4) in period 3. All animals were sacrificed at the end of month 8.

TABLE 2

Incidence of Gallstones and Cholesterol Crystals in Prairie Dogs at Sacrifice

Group number	Experimental design 1:2:3[a]	Gallstones		Cholesterol crystals	
		Animals	(%)	Animals	(%)
1	C:C:C	0/4[b]	(0)	0/4[b]	(0)
2	C:C:L	11/17[c]	(59)	10/17[c]	(65)
3	C:A:L	6/7	(86)	7/7	(100)
4	A:A:A	1/4	(25)	3/4	(75)
5	A:A:L	17/17	(100)	17/17	(100)
6	A:C:L	8/8	(100)	8/8	(100)

[a]1:2:3 represents the experimental periods. C, chow; A, alfalfa-corn; L, lithogenic diet.
[b]Differs significantly from groups 2, 3, 5 and 6 by chi square; $p < 0.02$.
[c]Differs significantly from groups 5 and 6 by chi square; $p < 0.01$.

diet had lithogenic indices which approached or exceeded 1.0. The highest lithogenic index was present in animals fed alfalfa-corn for periods 1 and 2 followed by the lithogenic diet in period 3 (lithogenic index, 1.58). Even though the lithogenic index for the group given only alfalfa-corn (group 4) averaged 0.81, the one animal in this group that had cholesterol gallstones was found to have a lithogenic index of 1.01.

Biliary bile acid composition was analyzed at sacrifice (Table 5). The amount of chenodeoxycholic acid in the chow-fed animals (group 1) was twice that for animals fed only alfalfa-corn (group 4). As expected from previous studies, feeding diets high in cholesterol led to an increase in the amount of chenodeoxycholic acid and a decrease in cholic acid. These changes were similar for groups 2, 3, 5 and 6. The amounts of lithocholic acid and deoxycholic acid were small (ranging from 0.3% to 0.8% and 0.7% to 2.6%, respectively).

TABLE 3

Cholesterol Concentrations and Enzyme Activities in Prairie Dogs

Group number	Experimental design 1:2:3[a]	Cholesterol			Hepatic HMG-CoA reductase pmoles/mg protein/min	Cholesterol 7α-hydroxylase pmoles/mg protein/min
		Liver mg/g	Plasma mg/dl	Bile mg/ml		
1	C:C:C	2.66 ± 0.09[b]	100 ± 3[c]	0.25 ± 0.02[d]	121 ± 23[e]	16 ± 3[f]
2	C:C:L	6.32 ± 0.52	690 ± 12	0.43 ± 0.05	26 ± 4	15 ± 1
3	C:A:L	7.24 ± 1.18	710 ± 14	0.25 ± 0.05	57 ± 7	10 ± 1
4	A:A:A	2.63 ± 0.18	100 ± 4[c]	0.15 ± 0.03[g]	269 ± 38[h]	13 ± 3
5	A:A:L	7.73 ± 0.73	1030 ± 12[i]	0.35 ± 0.05	68 ± 11	8 ± 1[j]
6	A:C:L	6.44 ± 0.49	700 ± 0	0.34 ± 0.07	35 ± 2	17 ± 1

[a]1:2:3 represents the experimental periods. C, chow; A, alfalfa-corn; L, lithogenic diet. Numbers are mean ± SEM (standard error of the mean).
[b,c,h]Differs from groups 2, 3, 5 and 6; $p < 0.01$.
[d]Differs from group 2; $p < 0.01$.
[e]Differs from groups 2–6; $p < 0.01$.
[f]Differs from group 3; $p < 0.01$.
[g]Differs from groups 2, 5 and 6; $p < 0.01$.
[i]Differs from groups 1–4 and 6; $p < 0.01$.
[j]Differs from groups 1, 2 and 6; $p < 0.01$.

TABLE 4

Biliary Lipid Composition at Sacrifice

Group number	Experimental design 1:2:3[a]	Mole %			Total lipid g/dl	Lithogenic index
		Cholesterol	Phospholipids	Bile acids		
1	C:C:C	4.0 ± 0.7[b]	11.8 ± 1.3	84.2 ± 2.0	9.2 ± 1.4	0.87 ± 0.03[b]
2	C:C:L	6.8 ± 0.7	15.6 ± 1.0	77.6 ± 1.0	8.4 ± 0.7	1.30 ± 0.11[c]
3	C:A:L	4.7 ± 0.2	14.2 ± 1.3	81.1 ± 1.5	7.5 ± 1.2	0.98 ± 0.07[d]
4	A:A:A	2.5 ± 0.3	6.4 ± 0.5[e]	91.1 ± 0.6	7.6 ± 1.0	0.81 ± 0.07[f]
5	A:A:L	7.7 ± 0.5	14.6 ± 1.3	77.7 ± 1.7	7.7 ± 0.9	1.58 ± 0.09
6	A:C:L	6.4 ± 0.4	15.7 ± 1.3	77.9 ± 1.5	7.1 ± 1.1	1.24 ± 0.07

[a]1:2:3 represents the experimental periods. C, chow; A, alfalfa-corn; L, lithogenic diet. Numbers are mean ± SEM.
[b]Differs from groups 2, 5, and 6; $p < 0.01$.
[c]Differs from group 5; $p < 0.05$.
[d]Differs from group 6; $p < 0.01$.
[e]Differs from groups 1–3, 5 and 6; $p < 0.01$.
[f]Differs from groups 2, 5 and 6; $p < 0.01$.

TABLE 5

Biliary Bile Acid Composition at Sacrifice

Group number	Experimental design 1:2:3[a]	Cholic acid %	Chenodeoxycholic acid %	Deoxycholic acid %	Lithocholic acid %	Allocholic acid %
1	C:C:C	80.6 ± 3.5[b]	17.8 ± 3.8[b]	1.0 ± 0.1	0.5 ± 0.1	0.1 ± 0.0[c]
2	C:C:L	42.5 ± 2.6	52.5 ± 2.6	2.0 ± 0.5	0.8 ± 0.1	2.2 ± 0.3
3	C:A:L	45.8 ± 3.1	51.2 ± 3.4	1.0 ± 0.4	0.4 ± 0.0	1.6 ± 0.2
4	A:A:A	88.1 ± 1.4[d]	9.2 ± 1.9[d]	0.7 ± 0.3	0.5 ± 0.1	1.5 ± 0.2
5	A:A:L	49.6 ± 4.1	44.8 ± 4.7	2.6 ± 0.7	0.7 ± 0.1	2.3 ± 0.4
6	A:C:L	52.7 ± 2.5	43.2 ± 2.9	1.7 ± 0.5	0.3 ± 0.0	2.1 ± 0.1

[a]1:2:3 represents the experimental periods. C, chow; A, alfalfa-corn; L, lithogenic diet. Numbers are mean ± SEM.

[b]Differs from groups 2 and 3; $p < 0.01$.

[c]Differs from groups 2-6; $p < 0.01$.

[d]Differs from groups 5 and 6; $p < 0.01$.

DISCUSSION

The prairie dog has been employed as a model of cholesterol cholelithiasis (8,22,23). In this species, it is relatively easy to induce cholesterol gallstones by administering a diet containing 0.4-1.2% cholesterol for a period of a few wk to several mo. Many investigators have reported a gallstone incidence of close to 100% when a semipurified basal diet was employed, to which cholesterol was added in the form of dried egg yolk powder and as crystalline cholesterol monohydrate (8,24). For unknown reasons, the combination of the semipurified diet plus cholesterol is not an ideal lithogenic diet in this model because it induces significant hepatotoxicity, characterized by bile duct proliferation, inflammatory reaction and portal fibrotic changes (10). When chow was substituted for the semipurified diet, liver damage was no longer observed, but the rate of stone formation was significantly decreased (24).

In our hands, gallstone formation in the prairie dog was not as consistent and reproducible as reported by others (8,22,23). We attributed this phenomenon to the fact that initially the animals used by most investigators including our group had been trapped in the wild and were quite heterogeneous. However, the present experiments demonstrate that even animals bred in captivity and in much better physical condition than trapped specimens exhibited a phenomenon not observed previously in prairie dogs: the response of the animals to the semipurified, high-cholesterol lithogenic diet depended on the dietary history of the animals or, more specifically, on the dietary regimen fed to the animals after weaning. The effect of prefeeding the prairie dogs different diets before administering the lithogenic diet became apparent when, in successive experiments of identical design, the incidence of cholesterol gallstones dropped from nearly 100% to 40-50%. In both studies, the lithogenic diet was identical (semipurified diet plus 0.4% cholesterol, for 8 wk), a diet previously shown to produce a high incidence of gallstones (8,10,23). Further investigation showed that the supplier of our animals had, for reasons of convenience, changed the diets of the prairie dogs during the six-month period after weaning. The diet given previously was an alfalfa-corn mixture that produced an adequate, high incidence of cholesterol gallstones when the lithogenic diet was administered. The diet fed subsequently was Purina rodent chow; after receiving this diet for six mo, followed by the lithogenic diet, the incidence of cholesterol stones was reduced to only 40-50%. We designed the present experiment to elucidate whether the incidence of gallstones was indeed affected by the dietary history, i.e., whether prefeeding the animals with either chow or alfalfa-corn would affect the incidence of gallstones when the animals were subsequently placed on the lithogenic diet.

The experimental protocol, a modified Latin square design, is shown in Table 1. The experiment was designed to show whether maintaining prairie dogs with an alfalfa-corn regimen, immediately after weaning (period 1) or after an intervening period on rodent chow or alfalfa-corn (period 2), affects gallstone formation and cholesterol metabolism after administration of the semipurified, cholesterol-containing lithogenic diet (period 3).

The data on the incidence of gallstones and biliary cholesterol crystals listed in Table 2 clearly demonstrate the difference between the chow diet and the alfalfa-corn regimen. As expected, the control animals fed chow throughout the three experimental periods had neither gallstones nor biliary cholesterol crystals. In contrast, administration of the alfalfa-corn diet during the entire eight-mo experimental period induced biliary cholesterol crystals in three out of four animals and gallstones in one animal. This result was unexpected, because this group (group 4) had not been exposed to any cholesterol in the diet. To confirm this effect of the alfalfa-corn diet, we maintained eight animals on a pelleted diet containing equal amounts of alfalfa and corn by weight. At the end of two mo, four of the animals had cholesterol gallstones and exhibited numerous cholesterol crystals in bile. Thus, it seems probable that this cholesterol-free diet is lithogenic in the prairie dog.

In general, the results listed in Table 2 confirm our previous observation that the incidence of cholesterol gallstones was reduced in animals raised and maintained

on chow before being exposed to the lithogenic diet (group 2) (24). In contrast, animals given alfalfa-corn, either before or after a period on chow, had an incidence of gallstones ranging from 86% (group 3) to 100% (groups 5 and 6). The animals in all three groups exhibited a 100% incidence of biliary cholesterol crystals. These data lead to the conclusion that the alfalfa-corn diet has both a long-term and a direct effect on one or several factors affecting gallstone formation.

We examined a number of parameters of cholesterol metabolism in an attempt to explain our data. On the cholesterol-free diets (groups 1 and 4), liver and serum cholesterol levels were within normal limits (Table 3) and the lithogenic indices were well below 1.0. However, the single animal of group 4 with gallstones had a lithogenic index of 1.01. The presence of cholesterol crystals in the gallbladder bile at sacrifice (group 4) suggests that during the experiment these biles must have been supersaturated and that nucleating agent(s) may have been present. It is known that cholesterol-containing lithogenic diets produce, in the prairie dog, increased secretion of mucus and this may serve as the nucleating factor (25). It also is noteworthy that feeding the cholesterol-free alfalfa-corn diet was associated with a very significant elevation of hepatic microsomal HMG-CoA reductase activity (269 units for group 4; 121 units for group 1 [chow]). It has been observed previously that this enzyme activity was elevated in human gallstone patients, and it has been suggested that increased hepatic HMG-CoA reductase activity is associated with an increased secretion of cholesterol into the bile (26,27). In rats, we determined that HMG-CoA reductase activity reflected hepatic cholesterol synthesis (28). At present, we have no mechanistic explanation for the stimulation of HMG-CoA reductase activity by the alfalfa-corn diet, but it seems likely that the very high fiber content inhibits cholesterol absorption. However, it also must be stated that, on the average, the alfalfa-corn group had the lowest cholesterol level in gallbladder bile of all the groups studied. Obviously, more detailed studies on the influence of these diets on hepatic cholesterol secretion are needed.

The effects of the cholesterol-containing lithogenic diet in animals previously maintained with alfalfa-corn again demonstrates the effect of dietary history on the development of cholesterol cholelithiasis. Only those prairie dogs receiving alfalfa-corn immediately after weaning (period 1) subsequently exhibited a 100% incidence of gallstones and cholesterol crystals (groups 5 and 6). It is of interest that animals maintained with chow immediately after weaning, followed by alfalfa-corn in period 2, also had a very high incidence of gallstones (group 3, 86%) in comparison with animals given only chow during periods 1 and 2 (group 2, 59%). Measurement of tissue-cholesterol concentration and hepatic HMG-CoA reductase activity again suggested a strong effect of the alfalfa-corn diet on cholesterol metabolism. The highest plasma cholesterol levels and HMG-CoA reductase activity were found in group 5 (alfalfa-corn fed during periods 1 and 2). The data in Table 3 further demonstrate that animals fed chow before being given the lithogenic diet had lower HMG-CoA reductase activities than those receiving alfalfa-corn (groups 2 vs 3 and 5 vs 6). The activities of hepatic cholesterol 7α-hydroxylase in these groups were low, as is usually the case in the prairie dog, but nevertheless it can be seen that the activity of this rate-determining enzyme of bile acid synthesis varied in the opposite direction of that shown by HMG-CoA reductase.

These studies demonstrate a phenomenon not previously reported in the prairie dog or other species, namely, that the nature of the diet fed early in life affects the susceptibility to a lithogenic diet at a later time. Other investigators have had variable success in attempting to condition a hypercholesterolemic response by feeding different diets early during the life cycle. In the rat, high-fat or high-carbohydrate diets did not appear to modify the subsequent response to a high-cholesterol diet (29). In contrast, in the guinea pig, the administration of cholestyramine (a bile acid sequestrant) to neonatal animals caused a dramatic increase in plasma cholesterol and cholesterol degradation in adult life (30). The latter experiment suggested the possibility that dietary changes in early life can affect the subsequent response to a cholesterolemic stimulus.

The specific role of alfalfa-corn in the present studies, of course, is not yet known. On the basis of earlier work, it seems likely that nonnutritive dietary fiber may be responsible for the "memory" effect on cholesterol (and/or biliary lipid) metabolism. For example, in rats given a single dose of [4-^{14}C]cholesterol, the highest fecal excretion of radioisotope was observed when the animals were maintained with a diet containing alfalfa and corn oil (31). This suggests that in those and the present experiments alfalfa might have a bile acid binding effect similar to that of cholestyramine (32). Indeed, when Kritchevsky and Story studied the binding of taurocholate by semipurified diets supplemented with different dietary fiber, they found that alfalfa bound about one-fifth as much bile salt as cholestyramine and one-third as much as colestipol. (The latter drug, like cholestyramine, is used clinically to bind bile acids and reduce serum cholesterol concentrations [32,33].) In vitro studies likewise have shown that alfalfa can bind significant amounts of taurocholate (34). Kritchevsky and Story have presented an excellent, concise summary of the effect of dietary fiber on cholesterol/bile acid binding, absorption and metabolism (32). In the present experiments, taurocholate is the major bile salt of the prairie dog, but is partially replaced by taurochenodeoxycholate in the presence of dietary cholesterol.

Thus, our data suggest that, in the prairie dog, early exposure to the high-fiber, alfalfa-corn diet increases bile cholesterol biosynthesis. This increased rate of synthesis presumably persists even after this diet has been replaced by a diet of reduced fiber content (chow). It is known from studies in man that increased hepatic cholesterol synthesis is associated with, although not necessarily the cause of, cholesterol cholelithiasis. Obviously, the exact mechanism operative in animals given high-fiber diets remains to be established and, in particular, its effect on biliary lipids and nucleating/antinucleating agents.

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REFERENCES

1. Tepperman, J., Caldwell, F.T., and Tepperman, H.M. (1964) *Am. J. Physiol. 206,* 628-634.

2. Pearlman, B.J., Bonorris, G.G., Phillips, M.J., Chung, A., Vimdalal, S., Marks, J.W., and Schoenfield, L.J. (1979) *Gastroenterology 77,* 634-641.
3. Dam, H., and Christensen, F. (1952) *Acta Pathol. Microbiol. Scand. 20,* 236-242.
4. Dam, H., Prange, I., and Søndergaard, E. (1972) *Z. Ernährungsw. 11,* 80-94.
5. Kritchevsky, D., Story, J.A., and Klurfeld, D.M. (1978) *Experientia 34,* 1328.
6. Kritchevsky, D., and Klurfeld, D.M. (1979) *Am. J. Clin. Nutr. 32,* 2174-2176.
7. Brenneman, D.E., Connor, W.E., Forker, E.L., and DenBesten, L. (1972) *J. Clin. Invest. 51,* 1495-1503.
8. Doty, J.E., DenBesten, L., Roslyn, J.J., Pitt, H.A., Kuchenbecker, S.L., and Porter-Fink, V. (1982) *Am. J. Surg. 143,* 48-54.
9. Doty, J.E., Pitt, H.A., Kuchenbecker, S.L., and DenBesten, L. (1983) *Gastroenterology 85,* 168-174.
10. Cohen, B.I., Singhal, A.K., Stenger, R.J., May-Donath, P., Finver-Sadowsky, J., McSherry, C.K., and Mosbach, E.H. (1984) *Hepatology 4,* 300-307.
11. Singhal, A.K., Cohen, B.I., Mosbach, E.H., Une, M., Stenger, R.J., McSherry, C.K., May-Donath, P., and Palaia, T. (1984) *J. Lipid Res. 25,* 539-549.
12. Cohen, B.I., Singhal, A.K., Stenger, R.J., May-Donath, P., Finver-Sadowsky, J., McSherry, C.K., and Mosbach, E.H. (1984) *Lipids 19,* 515-521.
13. Schoenfield, L.J., Lachin, J.M., the NCGS Steering Committee, and the NCGS Group (1981) *Ann. Intern. Med. 95,* 257-282.
14. Dowling, R.H., Gleeson, D., Ruppin, D.C., Murphy, G.M., and the British/Belgium Study Group (1984) in *Enterohepatic Circulation of Bile Acids and Sterol Metabolism* (Paumgartner, G., Stiehl, A., and Gerok, W., eds.), pp. 361-369, MTP Press Ltd., Lancaster, England.
15. Fisher, M.M., Roberts, E.A., Rosen, I.E., Shapero, T.F., Sutherland, L.R., Davies, R.S., Bacchus, R., and Lee, S.V. (1985) *Hepatology 5,* 102-117.
16. Leuschner, U. (1986) *Med. Klin. 81,* 217-222.
17. Juniper, K., and Burson, E.N. (1957) *Gastroenterology 32,* 175-211.
18. Carey, M.C. (1978) *J. Lipid Res. 19,* 945-955.
19. Kuroki, S., Cohen, B.I., Carey, M.C., and Mosbach, E.H. (1986) *J. Lipid Res. 27,* 442-446.
20. Snedecor, G.W. (1948) in *Statistical Methods,* pp. 54-81, 182-189, Iowa State College Press, Ames, IA.
21. Howell, J.H., Marsh, M.E., Thiel, E.S., and Holzbach, R.T. (1983) *Biochim. Biophys. Acta 753,* 32-39.
22. Holzbach, R.T., Corbusier, C., Marsh, M., and Naito, H.K. (1976) *J. Lab. Clin. Med. 87,* 987-998.
23. Chang, S.-H., Ho, K.-J., and Taylor, C.B. (1973) *Arch. Pathol. 96,* 417-426.
24. Cohen, B.I., Mosbach, E.H., McSherry, C.K., Stenger, R.J., Kuroki, S., and Rzigalinski, B. (1986) *Hepatology 6,* 874-880.
25. Li, Y.F., Moody, F.G., Weisbrodt, N.W., Zalewsky, C.A., Coelho, J.C.U., Senninger, N., and Gouma, D. (1986) *Surgery 100,* 900-904.
26. Angelin, B. and Einarsson, K. (1985) in *Regulation of HMG-CoA* (Preiss, B., ed.), pp. 281-320, Academic Press, New York, NY.
27. Angelin, B., Ewerth, S., and Einarsson, K. (1983) *J. Lipid Res. 24,* 461-468.
28. Raicht, R.F., Cohen, B.I., Shefer, S., and Mosbach, E.H. (1975) *Biochim. Biophys. Acta 388,* 374-384.
29. Beynen, A.C., Van Tintelen, G., and Katan, M.B. (1984) *Nutr. Rep. Int. 30,* 545-552.
30. Li, J.R., Bale, L.K., and Subbiah, M.T.R. (1979) *Atherosclerosis 32,* 93-98.
31. Kritchevsky, D., Tepper, S.A., and Story, J.A. (1974) *Nutr. Rep. Int. 9,* 301-308.
32. Kritchevsky, D., and Story, J.A. (1976) in *The Bile Acids* (Nair, P.P., and Kritchevsky, D., eds.), Vol. 3, pp. 201-209, Plenum Press, New York, NY.
33. Kritchevsky, D., and Story, J.A. (1974) *J. Nutr. 104,* 458-462.
34. Dreher, M.L. (1987) in *Handbook of Dietary Fiber,* pp. 213-222, Marcel Dekker, Inc., New York, NY.

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METHODS

Location of Methyl Branchings in Fatty Acids: Fatty Acids in Uropygial Secretion of Shanghai Duck by GC-MS of 4,4-Dimethyloxazoline Derivatives[1]

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2-Substituted 4,4-dimethyloxazolines (DMOX) have been found to be a useful alternative to the commonly used methyl esters for the localization of unsaturated bonds and other substituents in the fatty chain by mass spectrometry. The powerful directed fragmentation coupled with good gas chromatographic ability enables the structure elucidation of modified fatty acids in complex mixtures. Continuing our previous study, 76 out of a total of 86 fatty acids obtained from the preen gland wax of Shanghai duck now have been identified by gas chromatography-mass spectrometry (GC-MS) of their oxazoline derivatives. The identification was based on the interpretation of the mass spectra and comparison with the spectra and equivalent chain lengths (ECL) of the corresponding methyl esters. Main components of this lipid mixture are straight chain fatty acids (8.22%), and 2-, 4- or 6-monomethyl branched acids (53.69%), amounting to 61.91% of the total acid fraction. In addition, a large number of dimethyl-substituted fatty acids (31.4%) also have been found. Typical mass spectra, which are easily recognizable and highly specific for fatty acids substituted at various positions, are presented and classified according to the structural feature of the chain.
***Lipids 23,* 804–810 (1988).**

Wax produced by the uropygial gland of birds has been the subject of intensive investigations. It is generally accepted that this wax, mainly composed of monoesters of fatty acids and alcohols, protects the plumage against wet. However, some additional functions can be assumed from the very complex structure of the lipids, which are known to be highly species-dependent. The uniqueness of this waxy material manifests itself in the number and kind of carbon skeletal types, in the wide range of chain lengths, and in the unusual patterns of substitution. Although much has still to be explained, from these variations a new taxonomy of birds has evolved (1,2). By studying the structure of relevant components, one may gain clues to the understanding of the functions of these externally secreted lipids.

Since the pioneering contributions of Stenhagen and collaborators, the structure of these fatty acids has been established with the aid of mass spectrometry (3,4). The mass spectra of branched fatty acid methyl esters have been studied most thoroughly (5–8). Because most identifications depend upon rather subtle differences in mass spectral peak intensities, an independent means of vertification is adopted in the concept of equivalent chain length (ECL), which has been fully expounded by Nicolaides et al. (9,10).

A newer, more attractive approach involves the use of other fragmentation-directing derivatives. Of particular interest is the achievement of N-acyl pyrrolidine derivatives by Andersson (11), and pyridylmethyl esters by Harvey (12,13). The latter has led to the structure elucidation of a very complex mixture of branched (iso- and anteiso-) fatty acids obtained from the meibomian gland secretion of rats (14).

In our efforts to develop newer methods that would lead to a clear-cut demonstration of chain feature in mass spectra, a search for derivatives with even better fragmentation-directing ability was initiated. Our strategy was to incorporate the carboxylic acid group into a nitrogen-containing heterocycle followed by examining the 70 eV EI mass spectra of the resulting "hidden" carboxylic acids (15). 2-Substituted benzoxazoles (16) and 2-substituted 4,4-dimethyloxazolines (DMOX) (17) were finally chosen for further investigation. The latter represent a class of interesting structure with a basic nitrogen atom. They are readily prepared and purified, markedly stable towards most reagents, and capable of hydrolytic decomposition on heating in acidic medium to regenerate the starting material. By virtue of these properties, they have been recommended by Meyers (18,19) as a means for the protection of -COOH group. Long chain fatty acid derivatives of this type were first prepared by Gronowitz and coworkers (20,21), and the mass spectral fragmentation were briefly discussed therein. Detailed study in our laboratory has shown that while possessing promising mass spectrometric as well as gas chromatographic characteristics, DMOX are useful derivatives for exploring chain structure in complex mixtures, for example, double bond (17), cyclopropane ring (22), triple bond, cyclopentene ring, and oxygenated groups (Zhang, J.Y., Yu, Q.T., and Huang, Z.H., unpublished data).

This paper describes an extension of the oxazoline method to the structure determination of branched acids derived from the wax of the uropygial gland of the Shanghai white duck, which is common in this area and belongs to the common species of *Anas platyrhynchos.*

MATERIALS AND METHODS

Materials. All standard fatty acids were purchased from the Sigma Chemical Co. (St. Louis, MO) with a purity

[1]Chemical Modification in Mass Spectrometry 6. For preceding paper in this series, see Ref. 17.

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Abbreviations: AMP, 2-amino-2-methyl-propanol; DCC, dicyclohexylcarbodiimide; DMF-DMA, N,N-dimethyl formamide dimethyl acetal; DMOX, 4,4-dimethyloxazolines; ECL, equivalent chain lengths; GC-MS, gas chromatography-mass spectrometry.

greater than 99%. 2-Amino-2-methyl-propanol (AMP) was a product of Aldrich Chemical Co. (Milwaukee, WI) with a purity of 99%.

Preparation of the mixed fatty acids. After washing with cold ether to remove the contaminating triglycerides from surrounding tissues, four preen glands (9.0 g), excised from freshly killed (in April) Shanghai ducks, were homogenized and extracted with chloroform/methanol (1:1, v/v) at room temperature. The organic layer, after diluting with an equal volume of water, was separated, dried and evaporated. The residue was taken up in petroleum ether (b.p. 60–90 C) and evaporated to afford 0.6 g of waxy material. The latter was finally saponified (1 part of KOH in 1 part of water and 4 parts of methanol, 80 C, 2 hr) and worked up by usual means. Yield of the mixed fatty acid was 70 mg.

Preparation of n-nonadecanoic acid-3d₂. The preparation of this new labeled acid was carried out according to literature (23). A mixture of 1.5 g of sodium salt of stearic acid and 3 ml of D_2O containing 12 mg of NaOD was heated in sealed tube at 200 C for 96 hr. After cooling, the mixture was acidified with dilute HCl and extracted with 3 × 5 ml of diethyl ether. The ether solution was evaporated. The residue was recrystallized from acetone and gave 1.4 g (92%) of 18:0-2d₂. Deuterium incorporation was 88.4%, based on MS.

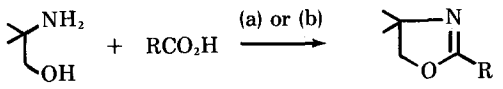
1.2 g of stearic acid-2d₂ was converted into its methyl ester by refluxing with 30 ml of 5% $BF_3 \cdot Et_2O/CH_3OH$ (v/v) solution for 0.5 hr. The methyl ester was separated by usual ways, dried, evaporated and dissolved in 30 ml of anhydrous diethyl ether. To this solution, 1.5 g of lithium aluminum hydride was added, the mixture then was refluxed for five hr. The excess hydride was decomposed by adding water, then the solution was acidified with 10% sulfuric acid and extracted with 3 × 10 ml of ether. The ether layer was washed with aqueous $NaHCO_3$ and water, dried and evaporated to leave 830 mg (73%) of stearyl alcohol-2d₂.

One g of methane sulfonyl chloride was added dropwise to a cooled (0 C) solution of 500 mg of stearyl alcohol-2d₂ in 7 ml of dry pyridine. The mixture was stirred five hr at room temperature. The residue was taken up in 10 ml of methylene chloride, washed with water and dried. Recrystallization from petroleum ether gave 590 mg (92%) of stearyl-2d₂-mesylate.

Four hundred mg of potassium cyanide was added to a solution of 590 mg of above mesylate in 10 ml of dimethyl sulfoxide. The mixture was heated at 120 C with stirring for two hr, then cooled and dissolved in 2 ml of methylene chloride. After washing with water and drying, evaporation gave 430 mg of n-nonadecanoyl nitril-3d₂ (91%).

A solution of 400 mg of above nitrile in a mixture of 2 ml of 90% potassium hydroxide and 2 ml of ethanol was refluxed for 48 hr. Acidification with hydrochloric acid and extraction with chloroform after removal of the solvent and recrystallization from acetone gave 300 mg (70%) of n-nonadecanoic acid-3d₂. Deuterium incorporation 86.6% based on MS.

Derivatization. The procedure described in our previous paper (17,22) was modified as follows. (a) Ten mg of AMP was added to 5 mg of the fatty acid mixture in a microreaction flask equipped with an air-condenser and a magnetic bar. The mixture was heated at 180 ± 5 C under



nitrogen for one to two hr. The cooled mixture was triturated with 1 N aq KOH and extracted with light petroleum ether (10 ml). The extract was allowed to pass through a small column packed with ca. 500 mg of silica gel, which was covered with 20 mg of anhydrous sodium sulfate, and eluted with 20 ml of the same solvent. The eluate was collected and evaporated to dryness in a rotary evaporator to furnish a pale-yellow oily substance. Yields were nearly quantitative. (b) Another preparation was based on a modification of Ref. 24. Five mg of dicyclohexylcarbodiimide (DCC) was added to a solution of 5 mg of fatty acid sample in 1 ml of dichloromethane. After stirring for 10 min, 5 mg of AMP was added. The mixture was allowed to react under stirring at room temperature for four hr. After filtration, the mixture was evaporated to dryness. The oily intermediate was dissolved in 1 ml of dry diethyl ether and treated with 500 μl of freshly distilled thionyl chloride (0 C, 0.5 hr; then room temperature, 0.5 hr), washed with ice-cold sodium carbonate solution, and worked up as described in (a).

Methyl esters and ECL measurement. Methyl esters were prepared by a "on-column" esterification procedure by dissolving about 1 mg of sample in 100 μl of N,N-dimethyl formamide dimethyl acetal (DMF-DMA) and directly injecting the mixture (ca. 0.5 μl) onto injector port of gas chromatograph for GC-MS analysis. ECL for component acids were calculated by Nicolaides' method (8).

Instruments. GC-MS analysis was carried out on a MAT 44S gas chromatograph-mass spectrometer equipped with a 29 m × 0.28 mm glass capillary column coated with SE-54 (cross-linked and bonded). Carrier gas flow was 1 ml He/min, split ratio 1:10. Both separator and ion source were maintained at 250 C, column temperature 240 C. Other parameters were: electron energy 70 eV, emission current 0.6 mA.

RESULTS AND DISCUSSION

By using this derivatization technique, 76 out of 86 individual components have been identified. These acids, either normal or branched, have chain lengths from C_6 to C_{18}. The gas chromatogram and results of analysis are presented in Figure 1 and Table 1, respectively.

High-quality mass spectra were produced from the resulting DMOX in GC-MS analysis. Interestingly, the mass spectra of these derivatives are closely similar to those of the corresponding N-acyl pyrrolidines in the region above *m/z* 100. They both exhibit rather simple fragmentation patterns that are highly characteristic with respect to the carbon skeleton of the chain. A marked difference is that oxazolines suffer less from interference and appear to show more prominent diagnostic peaks, thus rendering the latter, in particular those occurring at the high-mass end of the spectrum, more easily recognizable.

All normal chain fatty acid DMOX derivatives (Table 1) have been checked by comparing their mass spectra and retention times with those obtained from authentic samples. For illustration, the spectrum of the palmitic acid [16:0] derivative is presented in Figure 2a. Generally,

TABLE 1

Analytical Results by Derivatization Through DMOX and Methyl Esters

DMOX[a,b]				Methyl esters[c]			
Peak no.	% of total	Structure	Diagnostic ions *m/z* (%)	Peak no.	ECL:found (calcd)	Structure	Diagnostic ions *m/z* (%)
1	1.75	2-Me-6:0	127 (100), M183 (2.0)				
2	0.68	7:0					
3	0.17	8:1(2)					
4	2.26	2-Me-7:0	127 (100), M197 (1.7)	1	7.43 (7.28)	2-Me-7:0	88 (100), 99 (2.4), 101 (21.9), M158 (0.2)
5	1.28	4-Me-7:0	140 (3.7)*, M197 (0.2)	2	7.64 (7.51)	4-Me-7:0	74 (100), 87 (87.4), 101 (11.3), M158 (0.2)
6	3.90	2-Me-8:0	127 (100), M211 (1.4)	3	8.28 (8.28)	2-Me-8:0	88 (100), 101 (21.1), M172 (0.2)
7	3.87	4-Me-8:0	140 (2.8)*, M211 (0.3)	4	8.59 (8.51)	4-Me-8:0	87 (100), 99 (23), 115 (17.4), M172 (0.3)
8	2.79	2,6-Me_2-8:0	126 (14.2), 127 (100), 182 (0.3)*, M225 (0.2)	5	9.08 (9.00)	2,6-Me_2-8:0	88 (100), 96 (4.7), M186 (0.4)
9	2.24	2-Me-9:0	127 (100), M225 (1.1)	6	9.49 (9.28)	2-Me-9:0	88 (100), 101 (23.3), M186 (0.3)
10	1.36	4-Me-9:0	140 (1.8)*, M225 (0.4)	7	9.56 (9.51)	4-Me-9:0	87 (100), 113 (13.0), 129 (7.0), 143 (0.3), M186 (0.1)
11	0.77	2,6-Me_2-9:0	126 (12.8), 127 (100), 182 (0.7)*, M239 (0.8)	8	9.88 (9.75)	2,6-Me_2-9:0	88 (100), 101 (39.2), 110 (5.5), M200 (0.5)
12	1.26	10:0		9	10.0	10:0	
13	3.90	2-Me-10:0	127 (100), M239 (0.7)	10	10.35 (10.28)	2-Me-10:0	88 (100), 143 (4.7), 157 (3.1), M200 (0.5)
14	3.85	4-Me-10:0	140 (1.1)*, M239 (0.2)	11	10.58 (10.51)	4-Me-10:0	87 (100), 127 (8.8), 143 (6.9), 157 (0.3), M200 (0.1)
15	1.98	2,6-Me_2-10:0	126 (12.1), 127 (100), 182 (0.3)*, M253 (0.5)	12	10.85 (10.75)	2,6-Me_2-10:0	88 (100), 97 (11.7), 111 (0.8), 124 (5.3), M214 (0.6)
16	0.53	2,8-Me_2-10:0	126 (12.9), 127 (100), 210 (3.1)*, M253 (2.6)	13	11.07 (11.00)	2,8-Me_2-10:0	88 (100), M214 (0.5)
17	0.17	11:0		14	11.0	11:0	
18	1.77	6-Me-11:0	113 (100), 126 (70.4), 127 (98.0), 168 (1.9)*, M253 (1.5)	15	11.20	unidentified	
				16	11.34	unidentified	
19	2.41	4-Me-11:0	140 (0.8)*, M253 (0.2)	17	11.55 (11.51)	4-Me-11:0	87 (100), 141 (6.9), 157 (7.0), M214 (0.2)
20	0.49	2,6-Me_2-11:0	126 (12.2), 127 (100), 182 (0.2)*, M267 (0.5)	18	11.79 (11.75)	2,6-Me_2-11:0	88 (100), 97 (12.7), 111 (1.0), 138 (5.1), M228 (0.6)
21	0.36	4,8-Me_2-11:0	140 (1.0)*, 210 (1.0)*, M267 (0.2)	20	12.03 (12.0)	4,8-Me_2-11:0	87 (88.0), 155 (3.2), 171 (4.7), M228 (0.4)
22	0.51	12:0		19	12.0	12:0	
23	3.53	2-Me-12:0	127 (100), M267 (0.6)	21	12.38 (12.28)	2-Me-12:0	88 (100), 101 (30.0), 171 (2.2), 185 (1.7), M228 (0.5)
24	3.90	4-Me-12:0	140 (0.9)*, M267 (0.2)	22	12.55 (12.51)	4-Me-12:0	87 (100), 99 (8.2), 155 (6.1), M228 (0.3)
25	1.43	2,6-Me_2-12:0	126 (11.6), 127 (100), 182 (0.4)*, M281 (0.4)	23	12.79 (12.73)	2,6-Me_2-12:0	88 (100), 97 (12.1), 101 (39.0) 111 (1.1), 152 (4.5), M242 (0.6)
26	0.34	2,8-Me_2-12:0	126 (12.9), 127 (100), 210 (1.9)*, M281 (0.5)				
27	0.66	2,10-Me_2-12:0	126 (10.3), 127 (100), 238 (1.9)*, M281 (0.5)	25	13.03 (13.00)	2,10-Me_2-12:0	88 (100), 101 (38.9), 181 (4.2), M242 (1.1)
28	1.85	4,8-Me_2-12	140 (1.0)*, 210 (0.2)*, M281 (0.2)	24	12.83 (12.97)	4,8-Me_2-12:0	87 (100), 169 (1.1), 185 (2.2), M242 (1.1)
29	2.34	6-Me-13:0	113 (100), 126 (64.6), 127 (95.6), 168 (1.3)*, M281 (0.7)	26	13.0	unidentified	
				27	13.24	unidentified	
				28	13.34	2,x-Me_2-13:0	88 (100), M256 (0.2)
30	0.68	2,6-Me_2-13:0	126 (12.2), 127 (100), 182 (0.4)*, M295 (0.7)	31	13.76 (13.71)	2,6-Me_2-13:0	88 (100), 101 (32.2), 166 (3.8), M256 (0.4)
31	1.17	4-Me-13:0	140 (0.9)*, M281 (0.3)	30	13.52 (13.50)	4-Me-13:0	87 (100), 169 (4.2), 185 (9.6), M242 (0.4)
32	0.45	2,6,8-Me_3-12:0	126 (12.1), 127 (100), 182 (0.4)*, 224 (0.5)*, M295 (0.6)	29	13.41	2,6,x-Me_3-12:0	88 (100), 166 (4.1), M256 (0.2)
33	0.66	4,8-Me_2-13:0	140 (0.8)*, 210 (0.1)*, M295 (0.2)	32	13.93 (13.97)	4,8-Me_2-13:0	87 (100), 183 (2.6), 199 (3.8), M256 (0.5)
34	0.17	6,8-Me_2-13:0	127 (30.9), 168 (1.1)*, 210 (0.9)*, M295 (0.3)				
35	0.23	14:0		33	14.0	14:0	
36	2.49	2-Me-14:0	127 (100), M295 (0.6)	34	14.35 (14.28)	2-Me-14:0	88 (100), 101 (30.5), 199 (2.3), 213 (1.6), M256 (0.9)
				35	14.41	unidentified	

TABLE 1 (continued)

DMOX[a,b]				Methyl esters[c]			
Peak no.	% of total	Structure	Diagnostic ions *m/z* (%)	Peak no.	ECL:found (calcd)	Structure	Diagnostic ions *m/z* (%)
37	3.90	4-Me-14:0	140 (1.8)*, M295 (0.2)	36	14.51 (14.48)	4-Me-14:0	87 (100), 183 (5.1), 199 (8.6), M256 (0.6)
				37	14.57	unidentified	
38	1.87	2,6-Me_2-14:0	126 (13.4), 127 (100), 182 (0.6)*, M309 (0.3)	38	14.71 (14.70)	2,6-Me_2-14:0	88 (100), 97 (11.6), 180 (5.2), M270 (0.9)
39	0.66	2,8-Me_2-14:0	126 (12.5), 127 (100), 210 (0.4)*, M309 (0.5)	39	14.82 (14.70)	2,8-Me_2-14:0	88 (100), 157 (9.7), 185 (1.7), M270 (1.9)
				40	14.89	unidentified	
40	0.77	2,10-Me_2-14:0	126 (11.5), 127 (100), 238 (0.2)*, M309 (0.5)	42	15.04	2,x-Me_2-14:0	88 (100), M270 (1.3)
41	3.19	4,8-Me_2-14:0	140 (1.1)*, 210 (0.1)*, M309 (0.2)	41	15.00 (14.90)	4,8-Me_2-14:0	87 (100), 197 (2.5), 213 (12.2), M270 (0.5)
42	1.85	6,10-Me_2-14:0	127 (57.2), 168 (1.5)*, 238 (0.5)*, M309 (0.3)	43	15.19	unidentified	
				44	15.33	unidentified	
				45	15.37	unidentified	
43	1.19	4-Me-15:0	140 (5.7)*, M309 (0.3)	46	15.48 (15.48)	4-Me-15:0	87 (100), 197 (3.7), 213 (8.9), M270 (0.6)
44	1.75	6,8-Me_2-15:0	127 (23.8), 168 (1.2)*, 210 (0.4)*, M323 (0.5)	47	15.56 (15.32)	unidentified	
45	0.13	2,10-Me_2-15:0	126 (24.0), 127 (100), 238 (0.3)*, M323 (0.5)	48	15.70	2,x-Me_2-15:0	88 (100), M284 (0.4)
46	0.77	4,8-Me_2-15:0	140 (0.9)*, 210 (0.1)*, M323 (0.2)	49	15.85 (15.85)	4,8-Me_2-15:0	87 (100), 125 (2.1), 211 (2.3), M284 (0.2)
47	0.53	6,10-Me_2-15:0	127 (42.4), 168 (2.6)*, 238 (1.2)*, M323 (0.2)				
48	3.30	16:0		50	16.0	16:0	
49	1.53	2-Me-16:0	127 (100), M323 (0.4)				
50	0.17	4,8,10-Me_3-15:0	140 (4.7)*, 210 (0.5)*, 252 (0.2)*, M337 (0.2)	51	16.08	unidentified	
51	3.87	6-Me-16:0	113 (100), 126 (31.5), 127 (27.1), 168 (3.5)*, M323 (0.3)	52	16.40 (16.39)	6-Me-16:0	74 (100), 143 (32.3), 208 (3.7), M284 (0.7)
				53	16.48	unidentified	
				54	16.52	unidentified	
52	0.43	2,10-Me_2-16:0	126 (17.2), 127 (100), 238 (0.4)*, M337 (0.4)	55	16.72 (16.71)	2,10-Me_2-16:0	88 (100), 101 (40.9), 157 (12.0) M298 (0.8)
53	0.55	unidentified					
54	0.66	2,12-Me_2-16:0	126 (9.2), 127 (100), 266 (0.1)*, M337 (0.4)	56	16.84	2,x-Me_2-16:0	88 (100), M298 (1.4)
55	2.09	4,8-Me_2-16:0	140 (1.3)*, 210 (0.3)*, M337 (0.2)	57	16.92 (16.87)	4,8-Me_2-16:0	87 (100), 97 (10.2), 225 (1.7), 241 (9.3), M298 (0.6)
56	1.92	4,10-Me_2-16:0	140 (4.1)*, 238 (0.2)*, M337 (0.3)	58	17.21	4,x-Me_2-16:0	87 (100), 225 (1.9), 241 (8.3), M298 (0.6)
57	0.68	8,12-Me_2-16:0	196 (0.3)*, 266 (0.1)*, M337 (0.3)				
58	0.89	6,12-Me_2-16:0	127 (53.8), 168 (1.3)*, 266 (0.3)*, M337 (0.4)				
59	0.47	17:0		59	17.0	17:0	
60	0.83	6-Me-17:0	113 (100), 126 (47.7), 127 (22.0), 168 (1.7)*, M337 (0.4)	60	17.46 (17.39)	6-Me-17:0	74 (100), 143 (13.2), 208 (0.8), M298 (1.2)
61	0.85	4,8,x-Me_3-16:0	140 (3.4)*, 210 (0.2)*, M351 (0.1)	61	17.29	4,x,x-Me_3-16:0	87 (100), 255 (7.3), M312 (0.2)
62	0.57	6,8,x-Me_3-16:0	127 (24.4), 168 (1.6)*, 210 (0.9)*, M351 (0.1)	62	17.38	unidentified	
63	0.51	4,10,x-Me_3-16:0	140 (3.3)*, 238 (0.3)*, M351 (0.1)	63	17.54 (17.59)	4,x,x-Me_3-16:0	87 (100), 255 (7.3), M312 (0.2)
64	0.66	18:2 (9,12)		64		18:2	
65	1.96	18:1 (9)		65		18:1	
66	0.19	unidentified					
67	1.26	18:0		66	18.0	18:0	
68	0.29	4-Me-18:0	140 (2.9)*, M351 (0.1)				

*"Local minimum" in the general profile of the spectra.

[a]All saturated fatty acids show identical diagnostic peaks as in the corresponding standards.

[b]Components with amount less than 0.1% (9:1[3], 8:0, 2,4,x-Me_3-7:0, 6-Me-8:0, 10:1[3], 9:0, 12:1[6], 8-Me-10:0, 2,4-Me_2-10:0, 2,8-Me_2-11:0, 13:0, 2,8-Me_2-13:0, 2,10-Me_2-13:0, 15:0, 2,8-Me_2-15:0, 6,x-Me_2-15:0 and two unidentified peaks) are not given in the table.

[c]Peaks 19, 20; 24, 25; 29–31; 41, 42 of methyl esters emerged in different orders as the corresponding DMOX.

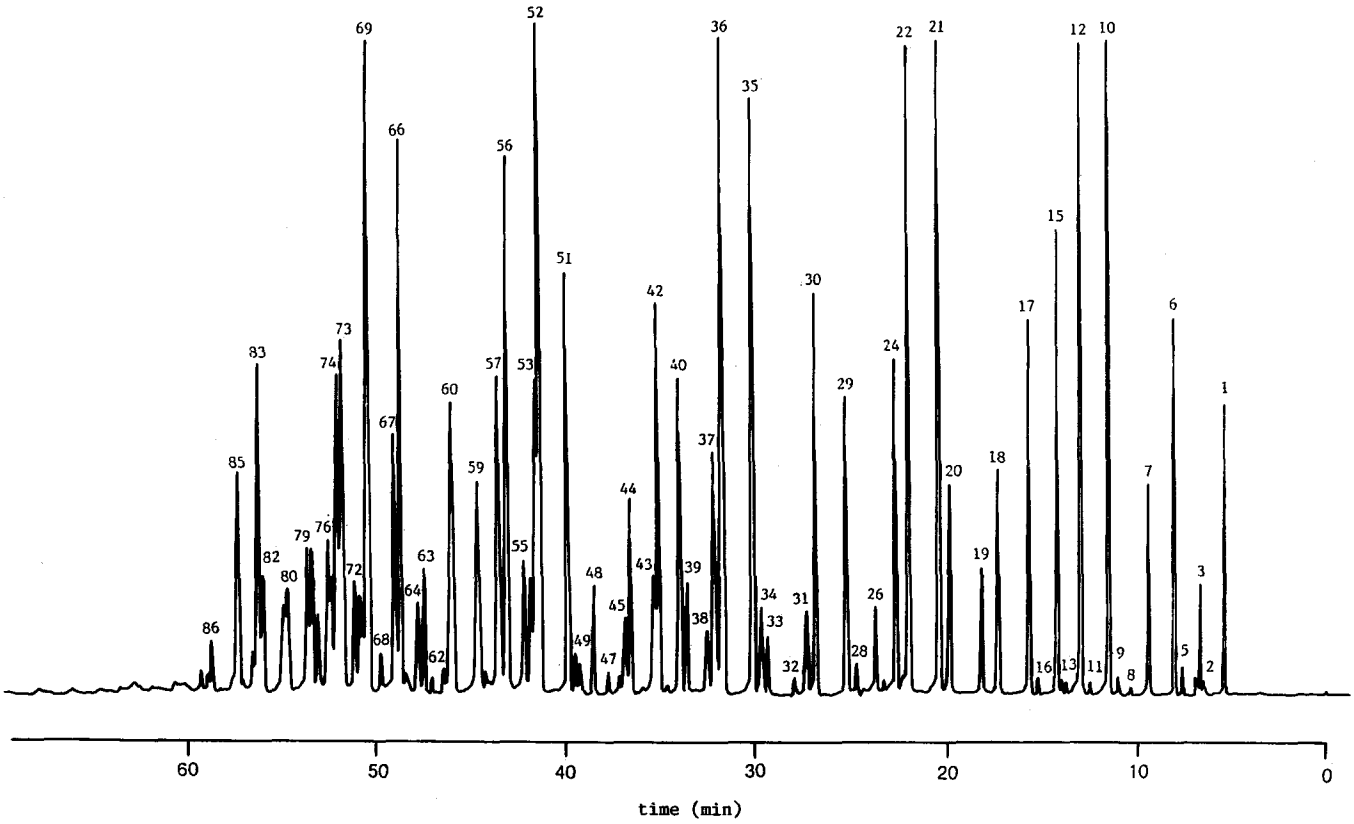


FIG. 1. Gas chromatogram of DMOX of fatty acid mixture obtained from the preen gland secretion of Shanghai white duck *(A. platyrhynchos)*.

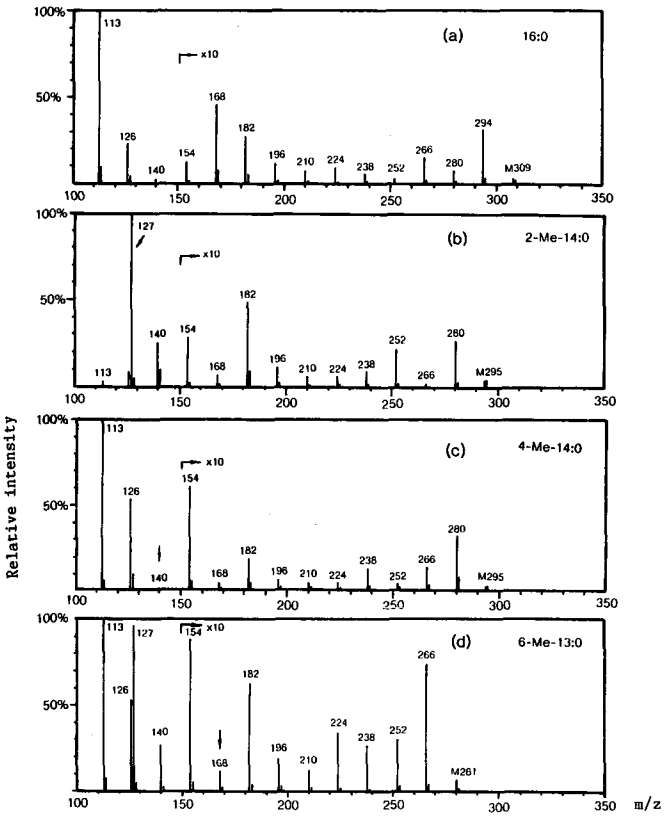


FIG. 2. (a–d) Typical mass spectra of DMOX of branched chain fatty acids.

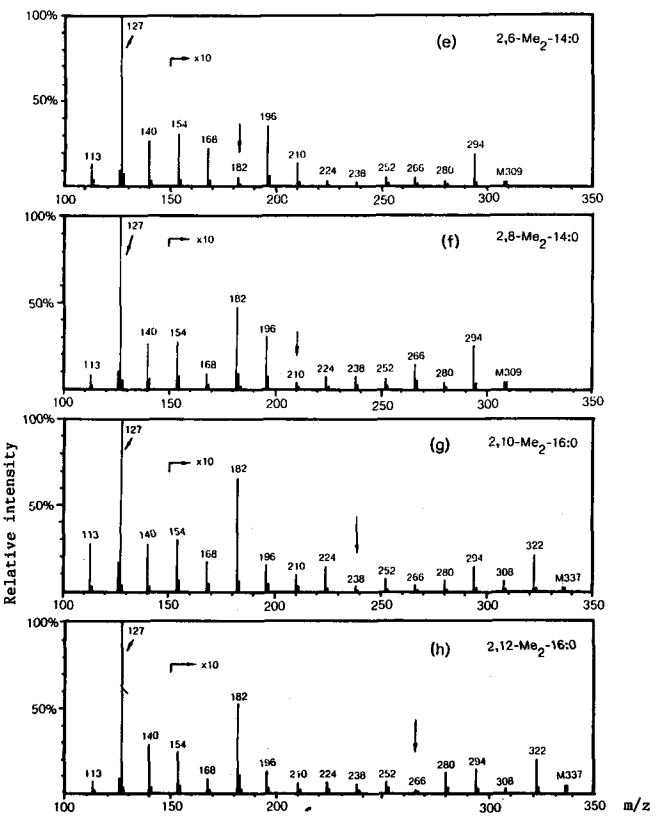
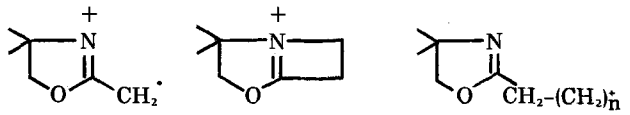


FIG. 2. (e–h) Typical mass spectra of DMOX of branched chain fatty acids.

two types of fragment ion peaks are displayed in the spectra of DMOX of saturated fatty acids: (i) an intense peak at *m/z* 113 (normally as base peak) is obtained as a result of the McLafferty rearrangement of 2-unsubstituted acids; and (ii) an even-mass homologous ion series at *m/z* 126 + 14n (n = 1, 2, 3, . . .) is formed by simple cleavage of the chain. This series, generally with a "local minimum" at C_5 (*m/z* 154) in straight chain homologs, distributes continuously up to the molecular ion region. The initial member, *m/z* 126, generated via cyclization-displacement reaction, frequently appears to be the most abundant ion within this series.



From the above statement, one can infer any variation in the peak profile may serve as a sign of chain-substitution. For example, the presence of a methyl branch is indicated by the existence of a newly created "local minimum" between two flanking ion clusters, whereupon the highest peaks of them are spaced by an interval of 28 mass units. Therefore, 2-methyl-substituted acid derivatives (see, for example, Fig. 2b) exhibit a base peak at *m/z* 127 (= 113 + 14) and a "local minimum" at *m/z* 168 in their spectra. Here a mass shift of 14 a.m.u. occurs as compared with the normal chain compound (Fig. 2a). Other substituted acids behave in like manner. This peak profile analysis is easy to handle and presents little uncertainty in structure assignment of fatty acids having a higher degree of substitution.

Similar to methyl esters, additional information may sometimes be provided by an elimination reaction resulting in the loss of two or three carbon atoms from within the chain (3,4). DMOX of a deuterated fatty acid, 19:0-3d_2, shows extrusion of carbon 2 and 3 of the original fatty acid as indicated by the ion M-31 (and M-45 for a three carbon loss) in the spectrum (Fig. 3).

Consequently, if a methyl branch is attached to either of these C-atoms (e.g., 2-Me, 4-Me, or di-Me substituted acids with the first branch at C-2 or C-4), the ion representing M-29 in case of 2-Me, or M-43 in the case of 4-Me, is seriously weakened, while the mass of the fragment lost from within the chain is correspondingly increased by 14 a.m.u. (compare, for example, M-29 in Fig. 2b, e–h; and M-43 in Fig. 2c, i and j).

The mass spectrometric characteristics of the fatty acids listed in Table 1 are classified according to the substitution pattern (Table 2). Typical mass spectra are presented in Figure 2.

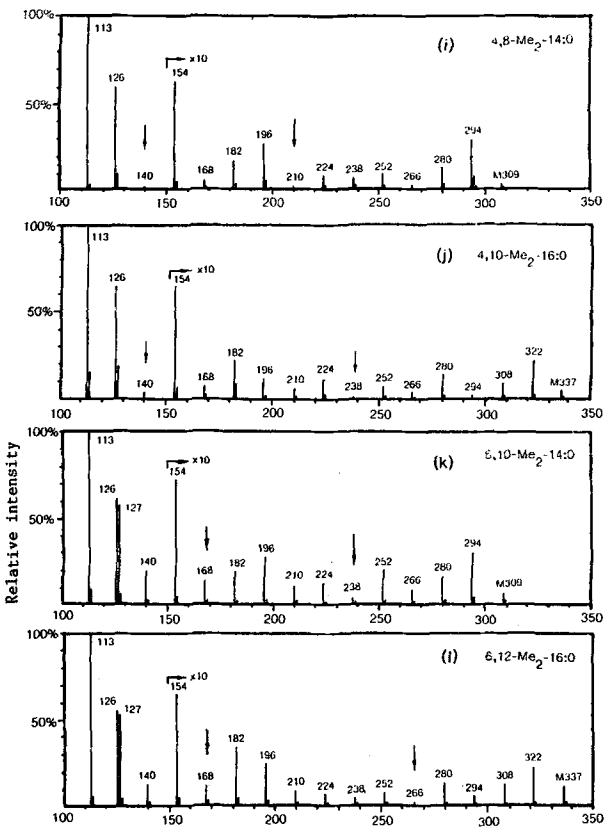


FIG. 2. (i–l) Typical mass spectra of DMOX of branched chain fatty acids.

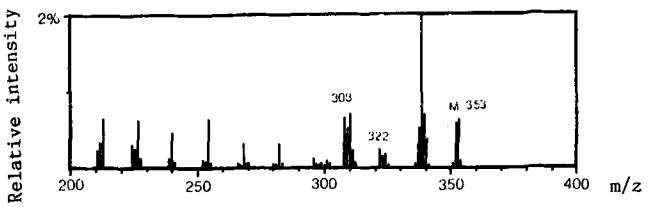


FIG. 3. Spectrum of DMOX from 19:0-3d_2.

TABLE 2

MS Characteristic Peaks of DMOX[a]

Fatty acid	Base peak *m/z*	2nd large *m/z*	Other characteristics *m/z*
Normal	113	126	154*
2-Me	127	140	168*
2,4-Me_2	127	140	low 113, 154*
2,6-Me_2	127	140	low 113, 168*, 182*
2,8-Me_2	127	140	low 113, 168*, 210*
2,10-Me_2	127	140	low 113, 168*, 238*
2,12-Me_2	127	140	low 113, 168*, 266*
4-Me	113	126	140*, 168*
4,8-Me_2	113	126	140*, 168*, 210*
4,10-Me_2	113	126	140*, 168*, 238*
6-Me	113	126/127	168*
6,8-Me_2	113	126/127	168*, 210*
6,10-Me_2	113	126/127	168*, 238*
6,12-Me_2	113	126/127	168*, 266*
8-Me	113	126	154*, 196*
8,12-Me_2	113	126	154*, 196*, 266*
12-Me	113	126	154*, 252*

[a] *m/z* 126 is accompanied by a strong satellite peak at *m/z* 127 in 6-Me series by an unexplored mechanism. Similar observation of *m/z* 127 occurred with DMOX of 6,7-methyleneoctadecanoic acid (22).
*"Local minimum" in the general profile of the spectra.

The structural assignment was further substantiated by GC-MS of methyl esters obtained from the same origin. Mass spectral data and ECL of individual methyl esters are presented in Table 1. Small, but acceptable differences between measured and calculated ECL values were observed and could be attributed to the change in experimental conditions. Identification by both DMOX and methyl esters agrees quite well except for certain minor components, which were not detected by the ester method. Generally, DMOX are rather volatile and a column temperature of ca. 10 C higher than that for the methyl esters is enough to achieve good separation. In addition, their relatively abundant diagnostic peaks and regular fragmentation make detection easy and the interpretation of spectra straightforward. The advantages of DMOX are obvious in cases of fatty acids having one or more than one branch located remote from the carboxylic acid end, such as 8- or 10-substitution. Under such circumstances, the utility of methyl esters is seriously hampered.

Uropygial gland waxes of the Peking duck, the common European mallard, the Rouen duck and the East Indian duck, all of which originate from *A. platyrhynchos*, were studied by several laboratories. Odham found and identified, from Peking duck wax, 16 normal-chain and monomethyl substituted fatty acids of C_6 to C_{11} (25) and from mallard duck wax, 10 C_6–C_{11} analogs (26). Of these, the waxes are composed mainly of 2-methyl and 4-methylhexanoic acids (about 74% of the total acids). A different picture is related to the waxes of the black East Indian ducks, which were shown by Bertelsen and Nguyen (27) to consist essentially of 4-methyl substituted C_6-, C_8-, C_{15}- and C_{18}-carboxylic acids. Kolattukudy et al. have studied in detail the wax esters of male mallard ducks and revealed dramatic seasonal variation of the components (28). The fatty acid profile (Table 3) of the Shanghai duck appears to be in fairly good agreement with the findings of Kolattukudy. Nevertheless, the presence of fatty acids with 6-methyl substituents was not known until now.

TABLE 3

Distribution Pattern of Fatty Acids from the Preen Gland Wax of Shanghai White Duck

Structure	% of total	Structure	% of total
Normal C_{7-18}	8.22	4,10-Me_2-C_{16}	1.92
2-Me-$C_{6-10,12,14,16}$	21.60	4,10,x-Me_3-C_{16}	0.51
2,4-Me_2-C_{10}	0.04	6-Me-$C_{8,11,13,16,17}$	8.87
2,4,x-Me_3-C_7	0.06	6,8-Me_2-$C_{13,15}$	1.92
2,6-Me_2-C_{8-14}	10.01	6,8,x-Me_3-C_{16}	0.57
2,6,x-Me_3-C_{12}	0.45	6,10-Me_2-$C_{14,15}$	2.38
2,8-Me_2-C_{10-15}	1.78	6,12-Me_2-C_{16}	0.89
2,10-Me_2-C_{12-16}	2.10	6,x-Me_2-C_{15}	0.11
2,12-Me_2-C_{16}	0.66	8-Me-C_{10}	0.06
4-Me-$C_{7-15,18}$	23.22	8,12-Me_2-C_{16}	0.68
4,8-Me_2-C_{11-16}	8.92	Unsaturated $C_{8-10,12,18}$	3.05[a]
4,8,10-Me_3-C_{15}	0.17	Unidentified	0.96
4,8,x-Me_3-C_{16}	0.85		

[a]Peaks #5, 8, 14, 23, 82 and 83 represent minor unsaturated acids, which have been identified according to the empirical rule of "12 mass interval" as described (17). These acids were probably originated from contaminations in gland sampling and handling.

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REFERENCES

1. Jacob, J. (1975) *J. Chromatogr. Sci. 13,* 415–422.
2. Jacob, J. (1976) in *Chemistry and Biochemistry of Natural Waxes* (Kolattukudy, P.E., ed.) pp. 93–146, Elsevier, Amsterdam.
3. Stenhagen, E. (1968) in *Analysis and Characterization of Oils, Fats and Fat Products* (Boekenoogen, H.A., ed.) pp. 1–44, Vol. 2, Ch. 1, Interscience, New York.
4. Odham, G., and Stenhagen, E. (1972) in *Biochemical Applications of Mass Spectrometry* (Wallar, G.R., ed.) pp. 211–228, Wiley-Interscience, New York-London-Sydney-Toronto.
5. Ryhage, R., and Stenhagen, E. (1960) *Arkiv. Kemi 15,* 291–315.
6. Meyerson, S., and Leitch, L.C. (1966) *J. Am. Chem. Soc. 88,* 56–60.
7. McCloskey, J.A. (1970) in *Topics in Lipid Chemistry* (Gunstone, F.D., ed.) Vol. 1, pp. 369–440.
8. Apon, J.M.B., and Nicolaides, N. (1975) *J. Chromatogr. Sci. 13,* 467–473.
9. Nicolaides, N., Apon, J.M.B., and Wang, D.H. (1976) *Lipids 16,* 279–308.
10. Nicolaides, N., and Apon, J.M.B. (1977) *Biomed. Mass Spectrom. 4,* 337–347.
11. Andersson, B.A. (1978) *Progr. Chem. Fats Other Lipids 16,* 279–308.
12. Harvey, D.J. (1982) *Biomed. Mass Spectrom. 9,* 33–38.
13. Harvey, D.J. (1984) *Biomed. Mass Spectrom. 11,* 340–347.
14. Harvey, D.J., Tiffany, J.M., Duerden, J.M., Pandher, K.S., and Mengher, L.S. (1987) *J. Chromatogr. Biomed. Appl. 58,* 253–263.
15. Huang, Z.H., Yu, Q.T., and Zhang, J.Y. (1987) *Acta Chim. Sinica 45,* 1077–1084; (1988) *Acta Chim. Sinica* (English edn.), 29–36.
16. Yu, Q.T., Zhang, J.Y., and Huang, Z.H. (1986) *Biomed. Environ. Mass Spectrom. 13,* 211–216.
17. Zhang, J.Y., Yu, Q.T., Liu, B.N., and Huang, Z.H. (1988) *Biomed. Environ. Mass Spectrom. 15,* 33–44.
18. Meyers, A.I., and Temple, D.J. (1970) *J. Am. Chem. Soc. 92,* 6644–6646.
19. Meyers, A.I., Temple, D.J., Nolen, R.L., and Mihelich, E.D. (1974) *J. Org. Chem. 39,* 2778–2783.
20. Svenson, R., and Gronowitz, S. (1982) *Chemica Scripta 19,* 149–153.
21. Herslof, M., and Gronowitz, S. (1983) *Chemica Scripta 22,* 230–235.
22. Zhang, J.Y., Yu, Q.T., and Huang, Z.H. (1987) *Shitzuryo Bunseki* (Japan) *35,* 23–30.
23. Aasen, A.J., Lauer, W.M., and Holman, R.T. (1970) *Lipids 5,* 869–877.
24. Fry, E.M. (1949) *J. Org. Chem. 14,* 887–894.
25. Odham, G. (1967) *Arkiv. Kemi 27,* 289–294.
26. Odham, G. (1964) *Arkiv. Kemi 22,* 417–445.
27. Bertelsen, O., and Nguyen, D.-N. (1982) *Chemica Scripta 20,* 234–236.
28. Kolattukudy, P.E., Bohnet, S., and Rogers, L. (1985) *J. Lipid Res. 26,* 989–994.

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Analysis of Free Malondialdehyde in Photoirradiated Corn Oil and Beef Fat Via a Pyrazole Derivative

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Malondialdehyde (MA) formed in linolenic acid, linoleic acid, corn oil and beef fat upon photoirradiation was determined by gas chromatography (GC). The MA produced was reacted with methylhydrazine to give 1-methylpyrazole and was subsequently analyzed on a GC equipped with a nitrogen-phosphorus specific detector and a fused silica capillary column. MA values determined by this method correspond to free or unbound MA levels. Linolenic and linoleic acids produced 867 μg MA/g and 106 μg MA/g, respectively. Oleic and stearic acids did not produce detectable levels of MA upon photoirradiation. Amounts of MA produced after eight hour irradiations of corn oil and beef fat were 56.24 μg/g and 25.01 μg/g, respectively. Some photoreaction products in irradiated corn oil also were identified as methylhydrazine derivatives.
Lipids 23, 811–814 (1988).

Malondialdehyde (MA) is well known as a product of lipid peroxidation and prostaglandin biosynthesis in animal tissues. It has been implicated in aging (1), mutagenesis (2,3,4), carcinogenesis (3,5) and radiation damage (6).

MA, as 2-thiobarbituric acid (TBA) reacting substances, has been found in steam distillates of lipid-rich foods such as roasted meats (7). In most cases, these MA levels were found to increase with cooking (7,8). A major source of MA is from peroxidation of polyunsaturated fatty acids (PUFA) containing more than two double bonds (9). Direct analysis of MA is very difficult because of its extreme reactivity. In addition, MA can be in hydrolyzable forms (i.e., protein or amino acid conjugates [10,11] or cyclic hydroperoxy intermediates [12]), which can complicate analysis of free MA.

Among methods for MA determination, the TBA method has been commonly used to give an indication of MA content in samples. This method is not specific for MA, and there are several examples that demonstrate its overestimation of MA (13–15). Several methods that give more accurate measures of bound MA in samples than the TBA assay have been reported. Some of these involve modification of the TBA assay (13,16). The TBA-MA adduct obtained from an extract of spinach leaves and purified on a Sephadex G-15 column resulted in a TBA value much less than that of the crude sample (13). More recently, the TBA-MA adduct has been measured after its separation by high performance liquid chromatography (HPLC) from other interfering TBA-complexes (16).

Among other methods, MA was reacted with 1-dansylhydrazine under acidic conditions and the resulting product, 1-dansylpyrazole, was determined by HPLC (15). Gas chromatography (GC) of MA as the tetramethyl acetal derivative prepared under acidic conditions also was reported (12). All of the above methods are generally expected to measure total (free plus bound) MA, since conditions in the assays are sufficient to hydrolyze bound MA.

Some methods for determination of free MA that have been reviewed include spectrophotometric, distillation and HPLC methods (16). Free MA in microsomal and urine samples has been directly determined by ion-pairing HPLC, but the structure of the eluate was not confirmed (17). Other HPLC procedures have been described by Esterbauer and coworkers (18) and by Lee and Csallany (19). In contrast to the HPLC methods, capillary GC methods offer greater efficiencies of separation and a variety of selective detectors not available in HPLC. A search of existing literature did not reveal any GC methods capable of free MA measurement.

In the present study, MA formed in lipid or fatty acid samples during photoirradiation was reacted with methylhydrazine. The reaction product, 1-methylpyrazole (1-MP), was analyzed by GC. This reaction occurs readily at neutral pH and at room temperature and can be used to measure free MA levels in the samples. Furthermore, because the reaction product contains nitrogen atoms, the highly sensitive and selective nitrogen-phosphorus detector (NPD) can be used.

MATERIALS AND METHODS

Synthesis of standard 1-MP. Malonaldehyde bis(dimethyl acetal) (10 g) (Aldrich Chemical Co., Milwaukee, WI) was added to 300 ml of 0.1 N HCl and the solution stirred for one hr at 50 C. After the solution was cooled to room temperature, 0.3 g methylhydrazine (Aldrich) in 30 ml water was added dropwise over a 10-min period with constant stirring. The reaction solution was stirred for an additional 30 min and then 22 ml of 2 N NaOH solution was added. The solution was stirred for an additional 10 min. Dichloromethane (40 ml) was added to the solution and the mixture shaken for five min. The dichloromethane layer was separated with a separatory funnel. The aqueous layer was extracted once more with dichloromethane (20 ml) and the extract was added to the original dichloromethane fraction. The combined solvent fractions were washed three times with 10 ml deionized water. After drying over anhydrous sodium sulfate, the extract was concentrated to 7 ml by distillation. The final colorless liquid product (1.86 g) was obtained from the concentrate by fractional distillation under reduced pressure (23 mm Hg) at 39 C. The spectral data of 1-MP are as follows: mass spectrum, *m/z* (relative intensity) 82 (M^+, 100), 54 (65), 53 (47), 41 (35); ^{1}H NMR (CDCl3) δ 3.80 (s, 3H), δ 6.12 (dd, 1H, J = 1.8, 2.2 Hz), δ 7.22 (d, 1H, J = 2.6 Hz), δ 7.38 (m, 1H).

Instrumental. A Hewlett-Packard Model 5880A GC equipped with a NPD and a 50 m × 0.25 mm i.d. bonded phase DB-WAX fused silica capillary column (J & W Scientific) was used for routine quantitative analysis. GC peak areas were calculated with a Hewlett-Packard 5880A

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Abbreviations: MA, malondialdehyde; 1-MP, 1-methylpyrazole; NMA, N-methylacetamide;PUFA, polyunsaturated fatty acid; TBA, 2-thiobarbituric acid; GC, gas chromatography; HPLC, high performance liquid chromatography; NPD, nitrogen-phosphorus detector.

series GC terminal. All photoreactions were performed at room temperature within a Rayonet RPR-100 reaction chamber fitted with eight medium-pressure UV lamps (λ = 300 nm). The GC-MS identification of methylhydrazine derivatives was performed with a ZAB-HS high resolution magnetic sector instrument equipped with a Hewlett-Packard Model 5790 GC. The ^{1}H NMR spectra were recorded on a Varian EM-390 NMR spectrometer.

Preparation of calibration curve for 1-MP analysis. A standard stock solution was prepared by adding 1 mg of 1-MP to 1 ml of dichloromethane. Various amounts of this stock solution (10, 20, 30, 40, 50, 60, 80 and 100 μl each) were measured into separate volumetric flasks. One hundred μl of GC internal standard N-methylacetamide (1 mg/ml dichloromethane) was added to each flask and each solution was adjusted to exactly 25 ml with dichloromethane. A 2μl aliquot of each solution was injected onto a GC equipped with an NPD. The GC peak area ratio of 1-MP to N-methylacetamide (NMA) was plotted against concentration of 1-MP. The calibration curve was linear for all of the above solutions.

Sample extracts in dichloromethane were treated exactly as were the standard solutions for the calibration curve. When necessary, samples were concentrated to 20 ml by fractional distillation. One hundred μl of NMA internal standard solution was added, and volumes adjusted to exactly 25 ml with dichloromethane. 1-MP concentrations were determined by comparison of peak area ratios (1-MP/NMA) with the standard calibration curve.

Determination of MA in photoirradiated fatty acids. Linolenic, linoleic, oleic and stearic acids of the highest available quality were purchased from Aldrich Chemical Co. and used without further purification. Each fatty acid (40 mg) was dissolved in 40 ml hexane in separate Pyrex containers. The hexane solutions were irradiated for four hr at 25 C. Separate 40 ml hexane solutions, each containing 40 mg of one of the fatty acids, were also prepared as nonirradiated controls. The photoreaction mixture or nonirradiated control was mixed with 20 μl of methylhydrazine and stirred for 10 min. The solution was twice extracted with 10 ml of 1 N HCl to recover 1-MP from the hexane phase. The combined aqueous extracts were mixed with 12 ml of 2 N NaOH solution. After stirring for 10 min, the 1-MP derivative was extracted into two 7-ml portions of dichloromethane. The combined dichloromethane extracts were further processed and analyzed for 1-MP as described at the end of the section on calibration curve preparation.

The recovery of 1-MP for the above method was performed as follows. Hexane (40 ml) containing 60 μg of 1-MP was extracted exactly as described above for irradiated and control fatty acid solutions beginning with the extraction with 1 N HCl. Amounts of recovered 1-MP were determined as described at the end of the section on calibration curve preparation.

Modified method for recovery of 1-MP from hexane solutions. The modified method was developed after the fatty acid studies in order to maximize recovery of 1-MP. Four hundred μl of 1-MP standard solution (3.18 mg/ml dichloromethane) was added to 100 ml of hexane and stirred for 30 min at room temperature. After addition of 40 ml of 1 N HCl, the solution was stirred for 20 min at room temperature. The hexane layer was separated and extracted once more with 40 ml of 1 N HCl. This HCl extract was added to the previous aqueous fraction and the combined extracts were washed with 15 ml of hexane. The aqueous fraction was mixed with 50 ml of 2 N NaOH (resulting pH was ca. 12) and 120 ml of deionized water, and then extracted with 45 ml of dichloromethane for four hr in a liquid-liquid continuous extractor. This final extract was further processed and analyzed for 1-MP as described at the end of the section on calibration curve preparation.

Recovery of 1-MP from malonaldehyde bis(dimethyl acetal) was performed as described below. Stock solutions containing 30–40 mg of the malondialdehyde precursor in 200 ml 0.1 N HCl were heated at 45–50 C to liberate free MA. Aliquots (0.5 ml) of the stock solution were added to 100 ml hexane. After addition of 50 μl methylhydrazine, the solution was extracted and analyzed for 1-MP as described above.

Quantitative analysis of MA formed from photolysis of corn oil and beef fat. Corn oil (2 g) or purified beef fat (2 g) (20) was dissolved in 250 ml of hexane. Solutions were irradiated in Pyrex containers for various time periods at 25 C. After irradiation, 100 μl of methylhydrazine was added to the solutions, which were then stirred for 30 min at room temperature. Recovery of the 1-MP was performed as described in the modified recovery method. Reaction solutions were mixed with 40 ml of 1 N HCl solution and stirred for 20 min. After separation of the aqueous layer, the remaining hexane layer was mixed with 40 ml of 1 N HCl solution and stirred for 20 min. The aqueous layer was added to the former aqueous fraction and the combined extracts were washed with 15 ml of hexane. The aqueous solution was then mixed with 50 ml of 2 N NaOH solution and stirred for 20 min. This solution was adjusted to 250 ml with distilled water and extracted with 45 ml of dichloromethane for four hr with a liquid-liquid continuous extractor. This final extract was further processed and analyzed for 1-MP as described at the end of the section on calibration curve preparation. The dichloromethane extract from irradiated corn oil was further analyzed for other methylhydrazine derivatives by GC–MS.

RESULTS AND DISCUSSION

In this work, free MA in samples was rapidly derivatized with methyl hydrazine to form 1-MP. Recovery of standard 1-MP from hexane solution by the method described for the determination of MA in photoirradiated fatty acids was 66% ± 8 (mean ± S.D., 3 determinations). To maximize sensitivity of the assay for MA, the extraction procedure used for MA determination in the fatty acid samples was modified. Recovery of standard 1-MP by the modified method was 94.4% ± 2.2 (mean ± S.D., 5 determinations). Recovery of MA (as the 1-MP derivative) prepared by the hydrolysis of malonaldehyde bis(dimethyl acetal) was 79.1% ± 2.1 (mean ± S.D., 4 determinations). The yield of 1-MP from reaction of MA and methyl hydrazine was calculated as 86% from the above recovery results. The reaction yield may be low because MA preparation in the present study may have generated side products (21) or the hydrolysis reaction may have been incomplete.

Photoirradiation of linolenic and linoleic acids for four hr produced 867 μg MA/g and 106 μg MA/g, respectively.

Nonirradiated control samples showed only 57 μg MA/g and 14 μg MA/g for linolenic and linoleic acids, respectively. Stearic and oleic acids did not produce detectable levels of MA upon photoirradiation.

The mechanism for formation of MA from linolenic acid radical via a prostaglandin-like endoperoxide intermediate has been proposed by Pryor and coworkers (9). The mechanism accounts for the observation that linolenic acid (3 double bonds) produced more MA than did linoleic acid (2 double bonds). It is possible that small amounts of MA could be generated from linoleic acid via such a mechanism, however the rate would be relatively slow since the stability of the bicycloendoperoxide intermediate formed from linoleic acid would be considerably less than that formed from linolenic acid (9).

Photosensitized oxidation of methyl linoleate has been reported to produce hydroperoxy cyclic peroxide intermediates that can be decomposed to produce volatile secondary oxidation products (22). These cyclic peroxide intermediates have been reported to form small amounts of MA by acid decomposition in methanol solution (12). It seems reasonable that such intermediates may have formed from linoleic acid in the presence of photosensitizer impurities. It is not known, however, if these intermediates decompose to form free MA under conditions of the present method.

Table 1 shows amounts of free MA determined from corn oil and beef fat irradiated (λ = 300 nm) in hexane solution for various time periods at 25 C. Previously reported fatty acid compositions for beef fat and corn oil also are given (23). Formation of MA from both corn oil and beef fat was linear with irradiation time. MA formation in corn oil was considerably higher than that in beef fat. This may be due to the difference in linoleic acid content based on the results of fatty acid photoirradiation experiments. The concentrations of linoleic acid in corn oil and beef fat have been reported as 57.3% and 3.7%, respectively (23). Among the fatty acids listed in Table 1, linolenic acid with its three double bonds should also produce MA, however its levels are similar in both beef fat and corn oil and may not account for the observed differences in MA production.

TABLE 1

Amounts of MA Determined in Irradiated Corn Oil and Beef Fat

Sample	Irradiation time (hr)	Amount (μg/g)[a]	Fatty acid composition (g/100 g sample)[b]	
Corn oil	2	8.52	Palmitic	10.7 ± 0.8
	4	16.91	Stearic	1.7 ± 0.2
	8	36.01	Palmitoleic	0.1 ± 0.1
	12	56.24	Oleic	24.6 ± 1.7
			Linoleic	57.3 ± 2.5
			Linolenic	0.8 ± 0.3
Beef fat	4	5.99	Palmitic	24.8 ± 3.5
	12	25.01	Stearic	18.7 ± 3.7
			Palmitoleic	4.7 ± 2.4
			Oleic	36.0 ± 4.9
			Linoleic	3.7 ± 2.0
			Linolenic	0.6 ± 0.6

[a]Average of two replicates.

[b]See reference 23. Values represent mean ± S.D.

Several methylhydrzine derivatives found in a reaction mixture of methylhydrazine and irradiated corn oil are reported in Table 2. Methylhydrazine reacted with carbonyl compounds and produced different derivatives. Figure 1 illustrates the general reactions of methylhydrazine with different carbonyl compounds. Monocarbonyl compounds such as formaldehyde react with methylhydrazine to form hydrazones. α,β-Unsaturated aldehydes such as acrolein form 1-methyl-2-pyrazolines and β-dicarbonyl compounds such as MA give 1-methylpyrazoles after reaction with methylhydrazine. Acrolein was recently shown to form in heated cooking oils and beef

TABLE 2

Methylhydrazine Derivatives Identified in Corn Oil Irradiated for Four Hr

Derivatives	Original compounds	GC peak area (%)
1-Methyl-2-pyrazoline	Acrolein	0.54
1-Methylpyrazole	Malondialdehyde	3.18
1,3 (or 5)-Dimethyl-2-pyrazoline	Acetylacetaldehyde	*a*
1-Methyl-5-propyl-2-pyrazoline	(E or Z)-2-hexenal	*a*
1,3,5-Trimethylpyrazole	2,4-Pentanedione	*a*
1-Methyl-5-butyl-2-pyrazoline	(E or Z)-2-heptenal	1.16
2-Pyrazoline derivative ($C_9H_{18}N_2$)	α,β-Unsaturated aldehyde ($C_8H_{14}O$)	*a*
2-Pyrazoline derivative ($C_9H_{18}N_2$)	α,β-Unsaturated aldehyde ($C_8H_{14}O$)	*a*
1-Methyl-5-pentyl-2-pyrazoline	(E or Z)-2-octenal	1.55
Pyrazole derivative ($C_8H_{14}N_2$)	β-Dicarbonyl ($C_7H_{12}O_2$)	1.24
Pyrazole derivative ($C_9H_{16}N_2$)	β-Dicarbonyl $C_8H_{14}O_2$)	14.03
Pyrazole derivative ($C_9H_{16}N_2$)	β-Dicarbonyl ($C_8H_{14}O_2$)	2.71

[a]Peak area less than 0.01%.

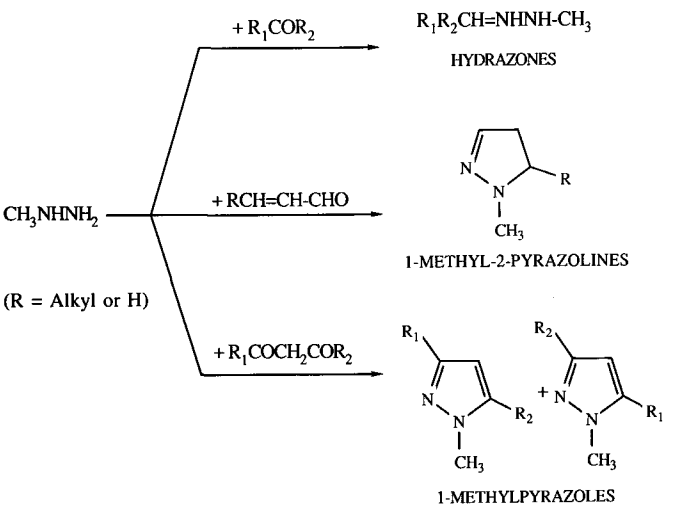

FIG. 1. Reactions of methylhydrazine with different classes of carbonyl compounds.

fat (24). 2,4-Dienoic aldehydes would be expected to produce 4-vinyl-1-methylpyrazoline derivatives, but were not observed in the MS analysis of photoirradiated corn oil. Further study must be done to determine if such compounds rearrange to form 4-alkyl-1-methylpyrazoles. Some compounds in Table 2 were deduced only as partial structures due to lack of authentic standards.

The method described in this work provides a means of measuring levels of free MA in certain samples. In pure lipid samples or other samples lacking amino acids and other compounds that may react with free MA, the method must provide a measure of the total MA. However, in many biological samples, MA may exist largely in a bound form which, under conditions of the present study, would not be released to form the MA derivative, thus underestimating total MA in these samples (10,16, 19). Other methods for MA analysis, such as that of Bird and Draper (16), may be more appropriate for measurement of MA in such samples because MA conjugates are hydrolyzed under conditions of the analysis.

In spite of limitations of the method presented here, there are certain advantages that allow a more specific measure of free MA. As mentioned, the mild conditions required for formation of 1-MP minimize the chance of hydrolysis or decomposition of bound forms of MA. Analysis of the MA derivative by capillary GC offers a higher degree of separation than can be accomplished by HPLC methods, thus minimizing the chance of coelution of interfering materials. Finally, methylhydrazine reacts rapidly with MA to form one major product, whereas other carbonyl compounds will react with methylhydrazine to form other derivatives. Further work is being performed to adapt the method for simultaneous analysis of trace levels of highly volatile or unstable compounds such as formaldehyde, acrolein and MA by capillary GC-NPD.

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REFERENCES

1. Munkres, K.D. (1976) *Mech. Ageing Dev. 5,* 171-191.
2. Mukai, F.H., and Goldstein, B.D. (1976) *Science 191,* 868-869.
3. Shamberger, R.J., Corlett, C.L., Beaman, K.D., and Kasten, B.L. (1979) *Mutat. Res. 66,* 349-355.
4. Basu, A.K., and Marnett, L.J. (1983) *Carcinogenesis 4,* 331-333.
5. Shamberger, R.J., Andreone, T.L., and Willis, C.E. (1974) *J. Natl. Cancer Inst. 53,* 1771-1773.
6. Packer, L., Deamer, D.W., and Heath, R.L. (1967) *Adv. Gerontol. Res. 2,* 77-120.
7. Siu, G.M., and Draper, H.H. (1978) *J. Food Sci. 43,* 1147-1149.
8. Shamberger, R.J., Shamberger, B.A., and Willis, C.E. (1977) *J. Nutr. 107,* 1404-1409.
9. Pryor, W.A., Stanley, J.P., and Blair, E. (1976) *Lipids 11,* 370-379.
10. Draper, H.H., McGirr, L.G., and Hadley, M. (1986) *Lipids 21,* 305-307.
11. Chio, K.S., and Tappel, A.L. (1969) *Biochem. 8,* 2827-2832.
12. Frankel, D.N., and Neff, W.E. (1983) *Biochim. Biophys. Acta 754,* 264-270.
13. Matsuo, T., Yoneda, T., and Itoo, S. (1984) *Agric. Biol. Chem. 48,* 1631-1633.
14. Csallany, A.S., Guan, M.D., Manwaring, J.D., and Addis, P.B. (1984) *Anal. Biochem. 142,* 277-283.
15. Hirayama, T., Yamada, N., Nohara, M., and Fukui, S. (1983) *J. Assoc. Off. Anal. Chem. 66,* 304-308.
16. Bird, R.P., and Draper, H.H. (1984) *Meth. Enzymol. 105,* 299-305.
17. Bull, A.W., and Marnett, L.J. (1985) *Anal. Biochem. 149,* 284-290.
18. Esterbauer, H., Lang, J., Zadravec, S., and Slater, T.F. (1984) *Meth. Enzymol. 105,* 319-328.
19. Lee, H.S., and Csallany, A.S. (1987) *Lipids 22,* 104-107.
20. Ohnishi, S., and Shibamoto, T. (1984) *J. Agric. Food Chem. 32,* 987-992.
21. Marnett, L.J., and Tuttle, M.A. (1980) *Cancer Res. 40,* 276-282.
22. Frankel, E.N., Neff, W.E., Selke, E., and Weisleder, D. (1982) *Lipids 17,* 11-18.
23. Sheppard, A.J., Iverson, J.L., and Weirauch, J.L. (1978) in *Handbook of Lipid Research* (Kuksis, A., ed.) Vol. 1, Fatty Acids and Glycerides, pp. 341-379, Plenum Press, New York.
24. Umano, K., and Shibamoto, T. (1987) *J. Agric. Food Chem. 35,* 909-912.

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COMMUNICATIONS

Phospholipid Analysis of Human Eosinophils: High Levels of Alkylacylglycerophosphocholine (PAF Precursor)

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The phospholipid composition and fatty acid profiles of human eosinophils were studied. Extremely high levels of ether phospholipids were found in this type of cell, such as alkylacylglycerophosphocholine (GPC) and alkenylacylglycerophosphoethanolamine (GPE); these two ether phospholipids accounted for about three-fourths of the choline and ethanolamine glycerophospholipids (CGP and EGP), respectively. Fatty acid analyses revealed that very large portions of arachidonic acid (20:4) were esterified to alkylacyl-GPC (92.0% in CGP) and alkenylacyl-GPE (86.6% in EGP), respectively. While high amounts of alkylacyl-GPC and the abundance of 20:4 in this ether phospholipid have been observed in other types of blood cells of various animals, these percentages for human eosinophils are the highest among such cells. These results suggest that alkyl and alkenyl ether phospholipids play essential roles in human eosinophils in various physiological and pathological conditions.
Lipids 23, 815–817 (1988).

It is well known that eosinophilia is an important diagnostic feature of several diseases such as parasitic infections (1–3) and bronchial asthma (4–7). Although the exact functions of eosinophils are not fully understood, it generally has been assumed that they are closely involved in the pathogeneses of such diseases. Eosinophils contain a number of granule cationic proteins that probably are involved in host defense reactions against parasitic infections (3,8) and tissue damage on inflammation (9). In addition, several chemical mediators, including platelet-activating factor (PAF) (10) and eicosanoids (11–13), were shown to be synthesized and released from eosinophils. These chemical mediators could play very important roles in active bronchial asthma (14,15). Despite the increased importance of eosinophils and their lipid mediators in allergic reactions, very little information is available as to the biochemical properties of the eosinophil membrane. For example, there are no data concerning the lipid composition or fatty acid distribution in eosinophils. Therefore, it is very important to investigate these points in detail to get a better understanding of the mechanism and the regulation of the biosynthesis of potent lipid mediators such as PAF (10) and leukotrienes (11–13) in eosinophils.

In this study, the phospholipid composition and fatty acid profiles of human blood eosinophils were investigated, and the highest levels of ether-containing choline glycerophospholipids (CGP) and ethanolamine glycerophospholipids (EGP) were observed as compared with other types of blood cells, whose phospholipid compositions had been analyzed.

MATERIALS AND METHODS

Chemicals. All chemicals were of reagent-grade, and solvents were distilled before use.

Cells. Human eosinophils were separated from peripheral blood of patients with eosinophilia by the Percoll (Pharmacia, Uppsala) discontinuous density gradient method (16). Briefly, leukocytes were isolated from heparinized blood by erythrocyte sedimentation. The cells were washed twice in isotonic buffer (Ca^{2+}, Mg^{2+}-free, pH 7.4) containing 10 mM ethylenediamine tetraacetic acid (EDTA) and 0.03% of human serum albumin, and then suspended in an isotonic Percoll solution (density, 1.070 g/ml). Isotonic Percoll solutions (1.100, 1.090, 1.085 and 1.080 g/ml) were layered in plastic tubes, and then the leukocytes in a Percoll solution were laid on top of the gradients. The tubes were centrifuged at 1600 × g for 20 min. The eosinophil and neutrophil fractions were collected and washed, respectively. The purity of eosinophils was 93%–97% and that of neutrophils >97%, as judged on morphological assessment (toluidine blue and light-green staining). The contaminating cells were mainly neutrophils or eosinophils, respectively.

Extraction and fractionation of lipids. Lipids were extracted by the method of Bligh and Dyer (17). Individual phospholipids were separated by two-dimensional thin layer chromatography (TLC) (18). Lipid spots were detected under ultraviolet light after spraying with primuline. Lipid phosphorus was estimated as described by Rouser et al. (19). CGP and EGP were eluted from the silica gel with chloroform/methanol/water (1:2:0.8, v/v/v) and extracted by the method of Bligh and Dyer (17).

Separation of alkenylacyl, alkylacyl and diacyl phospholipids. Alkenylacyl, alkylacyl and diacyl CGP and EGP were separated as 1,2-diradyl-3-acetylglycerol derivatives as described (18). The amounts of alkenylacyl, alkylacyl and diacyl compounds were estimated from their fatty acyl contents, using methyl heptadecanoate as an internal standard.

Determination of fatty acid compositions. Fatty acids were analyzed as methyl esters by gas liquid chromatography (GLC) as described (18).

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Abbreviations: CGP, choline glycerophospholipids; ECF-A, eosinophil chemotactic factor of anaphylaxis; EDTA, ethylenediamine tetraacetic acid; EGP, ethanolamine glycerophospholipids; FMLP, formylmethionylleucylphenylalanine; GPC, glycerophosphocholine; PAF, platelet-activating factor; GLC, gas liquid chromatography; TLC, thin layer chromatography.

TABLE 1

Phospholipid Composition of Human Blood Eosinophils[a]

Phospholipids	% of total phospholipids[b]
Choline glycerophospholipid	34.7 ± 2.5
Ethanolamine glycerophospholipid	27.7 ± 3.8
Sphingomyelin	19.8 ± 2.1
Serine glycerophospholipid	9.2 ± 1.6
Inositol glycerophospholipid	4.7 ± 0.6
Others	4.0 ± 2.0

[a]The mean percentages are for different samples (n = 6).

[b]The values were calculated on the basis of lipid phosphorus.

TABLE 2

Class Compositions of Choline and Ethanolamine Glycerophospholipids of Human Blood Eosinophils and Neutrophils

	Eosinophils[a]		Neutrophils[b]	
Class	CGP	EGP	CGP	EGP
Alkenylacyl	4.1 ± 2.6	72.7 ± 11.0	9.1	70.0
Alkylacyl	75.5 ± 4.4	6.1 ± 3.1	45.0	10.1
Diacyl	20.4 ± 5.9	21.2 ± 8.1	45.9	19.9

[a]The mean percentages are for different samples (n = 4).

[b]The mean percentages are for different samples (n = 2).

The values were calculated from the quantities of acyl moieties in each lipid class.

RESULTS AND DISCUSSION

Phospholipid composition. Table 1 shows the phospholipid composition of human blood eosinophils. The amount of lipid phosphorus was calculated to be 3.46 μg/10^7 cells. The predominant components were CGP and EGP. These results are generally consistent with the results for human neutrophils (20).

Amounts of ether-containing and diacyl phospholipids. Table 2 shows the class compositions of CGP and EGP of eosinophils and neutrophils. The class compositions of neutrophils were essentially the same as that reported by Mueller et al. (20). In eosinophils, an extremely high level of alkylacyl compounds was found in CGP. EGP contained a large amount of alkenylacyl compounds. The amounts of diacyl analogues were rather small in both CGP and EGP. The levels of alkenylacyl compounds in CGP and alkylacyl compounds in EGP were very low. We and some other investigators (18,20,21) have found that several types of white blood cells, such as neutrophils, macrophages and lymphocytes, contain very high amounts of alkylacyl-glycerophosphocholine (GPC) (16%–50% of CGP), which now is considered to be the stored precursor form of a potent lipid mediator, PAF (22). For example, 45 or 50% of CGP was accounted for by alkylacyl compounds in human blood neutrophils (Table 2, 21). Thus, the relative proportion of alkylacyl-GPC in human eosinophils is the highest, as compared with those in other types of white blood cells and other mammalian tissues, as far as we know (21). Although the biological roles of alkylacyl-GPC have not been fully elucidated, the extremely high proportion of alkylacyl-GPC in eosinophils could be favorable for the synthesis and release of PAF from them. In fact, increased acetyltransferase activity has already been observed in eosinophils stimulated with eosinophil chemotactic factor of anaphylaxis (ECF-A), C5a, formylmethionylleucylphenylalanine (FMLP) and Ca^{2+}-ionophore, A23187 (10).

Fatty acid compositions of CGP and EGP. The fatty acid compositions of CGP and EGP of human eosinophils and neutrophils are shown in Table 3. The fatty acid compositions in alkenylacyl-GPC and alkylacyl-glycerophosphoethanolamine (GPE) were not shown because of their very small amounts. The predominant fatty acids at the 2-position of alkylacyl-GPC were 20:4, 18:1 and 16:0, and those in the case of alkenylacyl-GPE 20:4, 22:5, 18:1 and 22:6. On the other hand, the relative proportions of C_{20} and C_{22} polyunsaturated fatty acids in diacyl-GPC and GPE were considerably lower. These fatty acid profiles of human eosinophils are similar to those of human neutrophils (Table 3). However, the percentage of 20:4 in alkylacyl-GPC in human eosinophils is ca. two times higher than that in human neutrophils.

Figure 1 shows the distribution of 20:4 in CGP and EGP in human eosinophils. Very large portions of 20:4 were found in alkylacyl compounds in CGP (92.0%) and alkenylacyl compounds in EGP (86.6%). These results indicate that most of the 20:4 in human eosinophils is stored in ether-containing phospholipids, which suggests the important roles of alkylacyl-GPC and alkenylacyl-GPE in the storage of 20:4.

High amounts of 20:4 in alkylacyl-GPC and alkenylacyl-GPE also have been observed in rabbit alveolar macrophages (18) and human neutrophils (20). We have already suggested that the high accumulation of 20:4 in ether phospholipids is due to the selective mobilization of 20:4 from diacyl-GPC to alkyl and alkenyl ether phospholipids in rabbit alveolar macrophages (23). These ether phospholipids could be one of the main sources of 20:4 in these cells upon stimulation. Several investigators have already demonstrated the release of considerable amounts of 20:4 from alkylacyl-GPC in various types of white blood cells upon stimulation with a variety of agents (24–27). It is very likely that the rapid release of 20:4 from this ether

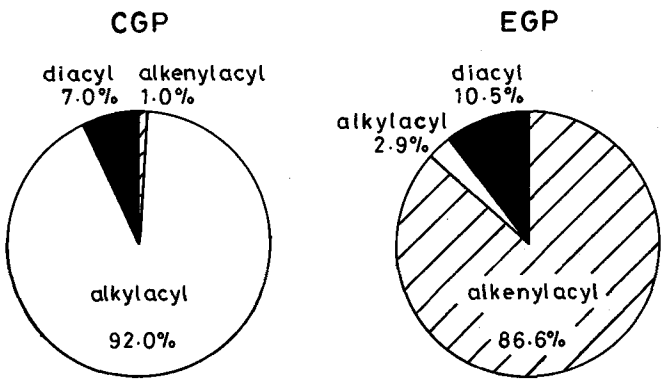

FIG. 1. Distribution of arachidonic acid among the lipid classes of choline and ethanolamine glycerophospholipids in human blood eosinophils. The values were calculated from the quantities of arachidonic acid in each lipid class.

TABLE 3

Fatty Acid Compositions of Choline and Ethanolamine Glycerophospholipids of Human Blood Eosinophils and Neutrophils

Fatty-chain	Eosinophils[a]				Neutrophils[b]			
	CGP		EGP		CGP		EGP	
	Alkylacyl	Diacyl	Alkenylacyl	Diacyl	Alkylacyl	Diacyl	Alkenylacyl	Diacyl
16:0	17.3 ± 1.2	36.8 ± 2.8	2.6 ± 2.1	10.8 ± 3.0	22.6	27.1	3.3	10.1
18:0	0.4 ± 0.3	15.2 ± 1.6	1.9 ± 1.6	36.7 ± 3.4	0.7	15.2	1.6	34.2
18:1	19.8 ± 3.0	32.6 ± 4.7	14.6 ± 4.5	28.9 ± 2.3	33.3	41.1	36.4	41.0
18:2	10.2 ± 2.8	10.4 ± 2.7	7.2 ± 2.1	3.2 ± 1.2	28.8	11.9	16.7	6.7
18:3 + 20:1	0.3 ± 0.2	0.9 ± 0.9	1.3 ± 1.3	1.3 ± 0.2	n.d.	2.6	0.6	2.1
20:3n-6	2.3 ± 1.5	0.1 ± 0.2	3.1 ± 0.8	n.d.	0.9	0.4	3.8	n.d.
20:4	23.3 ± 1.9	3.4 ± 1.0	36.2 ± 2.4	8.9 ± 0.7	11.7	1.5	26.7	5.9
22:4	5.7 ± 0.7	0.2 ± 0.4	5.1 ± 0.7	0.7 ± 0.5	0.2	n.d.	0.9	n.d.
22:5n-3	11.7 ± 1.5	n.d.	15.3 ± 3.8	5.2 ± 1.0	0.9	0.1	4.9	n.d.
22:6	9.0 ± 0.5	0.4 ± 0.2	12.6 ± 2.0	4.3 ± 2.0	0.9	0.1	5.0	n.d.

[a]The mean percentages are for different samples (n = 4).

[b]The mean percentages are for different samples (n = 2).

Fatty acids were analyzed as methyl esters by GLC. The fatty acid composition of diacyl subclass reflects both the *sn*-1 and *sn*-2 positions, whereas those of ether subclasses represent only the *sn*-2 position. n.d., Not detected.

phospholipid also takes place in eosinophils. Although the hydrolysis and release of 20:4 from stimulated eosinophils have not been studied in detail, it has been established that the generation of 5-lipoxygenase products, especially LTC_4, occurs in stimulated eosinophils (11-13). The results of this study indicate that significant portions of these lipoxygenase products probably originate from 20:4 derived from alkylarachidonoyl-GPC, a common precursor for PAF and eicosanoids. Although the biochemical features of alkylacyl-GPC have been studied in detail in rabbit alveolar macrophages (23,24), there is little information on that in human eosinophils. Further studies will reveal the dynamic and metabolical aspects of alkylacyl-GPC, and their biological significance in human eosinophils.

REFERENCES

1. Mahmoud, A.A.F., Warren, K.S., and Peters, P.A. (1975) *J. Exp. Med. 142,* 805-813.
2. Vadas, M.A., David, J.R., Butterworth, A.E., Pisani, N.T., and Siongok, T.A. (1979) *J. Immunol. 122,* 1228-1236.
3. Butterworth, A.E., and David, J.R. (1981) *N. Engl. J. Med. 304,* 154-156.
4. Lowell, F.C. (1967) *J. Am. Med. Assoc. 202,* 875-878.
5. Franklin, W. (1974) *N. Engl. J. Med. 290,* 1469-1472.
6. Guputa, S., Frenkel, R., Rosenstein, M., and Grieco, M.H. (1975) *Clin. Exp. Immunol. 22,* 438-445.
7. Horn, B.R., Robin, E.D., Theodore, J., and Van Kessel, A. (1975) *N. Engl. J. Med. 292,* 1152-1155.
8. Butterworth, A.E., Wassom, D.L., Gleich, G.J., Loegering, D.A., and David, J.R. (1979) *J. Immunol. 122,* 221-229.
9. Frigas, E., Loegering, D.A., and Gleich, G.J. (1980) *Lab. Invest. 42,* 35-43.
10. Lee, T-C., Lenihan, D.J., Malone, B., Roddy, L.L., and Wasserman, S.I. (1984) *J. Biol. Chem. 259,* 5526-5530.
11. Elsas, P., Lee, T.H., Lenzi, H.L., and Dessein, A.J. (1987) *Ann. Inst. Pasteur Immunol. 138,* 97-116.
12. Taniguchi, N., Mita, H., Saito, H., Yui, Y., Kajita, T., and Shida, T. (1985) *Allergy 40,* 571-573.
13. Bruynzeel, P.L., Kok, P.T., Hamelink, M.L., Kijne, A.M., and Verhagen, J. (1985) *FEBS Lett. 189,* 350-354.
14. Wardlaw, A.J., Moqbel, R., Cromwell, O., and Kay, A.B. (1986) *J. Clin. Invest. 78,* 1701-1706.
15. Page, C.P., Archer, C.B., Paul, W., and Morley, J. (1984) *Trends Pharmacol. Sci. 5,* 239-241.
16. Gärtner, I. (1980) *Immunology 40,* 133-136.
17. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
18. Sugiura, T., Nakajima, M., Sekiguchi, N., Nakagawa, Y., and Waku, K. (1983) *Lipids 18,* 125-129.
19. Rouser, G., Siakotos, A.N., and Fleischer, S. (1966) *Lipids 1,* 85-86.
20. Mueller, H.W., O'Flaherty, J.T., Greene, D.G., Samuel, M.P., and Wykle, R.L. (1984) *J. Lipid Res. 25,* 383-388.
21. Sugiura, T., and Waku, K. (1987) in *Platelet-Activating Factor and Related Lipid Mediators* (Snyder, F., ed.) pp. 55-85, Plenum Press, New York.
22. Albert, D.H., and Snyder, F. (1983) *J. Biol. Chem. 258,* 97-102.
23. Sugiura, T., Katayama, O., Fukui, J., Nakagawa, Y., and Waku, K. (1984) *FEBS Lett. 165,* 273-276.
24. Nakagawa, Y., Kurihara, K., Sugiura, T., and Waku, K. (1986) *Biochim. Biophys. Acta 876,* 601-610.
25. Swendsen, C.L., Ellis, J.M., Chilton, F.H., O'Flaherty, J.T., and Wykle, R.L. (1983) *Biochem. Biophys. Res. Commun. 113,* 72-79.
26. Albert, D.H., and Snyder, F. (1984) *Biochim. Biophys. Acta 796,* 92-101.
27. Billah, M.M., Bryant, R.W., and Siegel, M.I. (1985) *J. Biol. Chem. 260,* 6899-6906.

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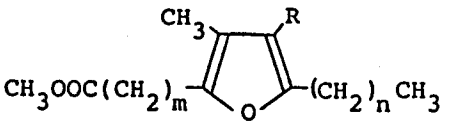
ERRATA

In the paper "The Composition of Furan Fatty Acids in the Crayfish," by Kazuo Ishii et al., Vol. 23, No. 7, pp. 694–700, there was an omission of one scheme in each of Tables 1 and 2.

TABLE 1

Furan Fatty Acid Composition of Sterol Esters[a] from Crayfish Hepatopancreas

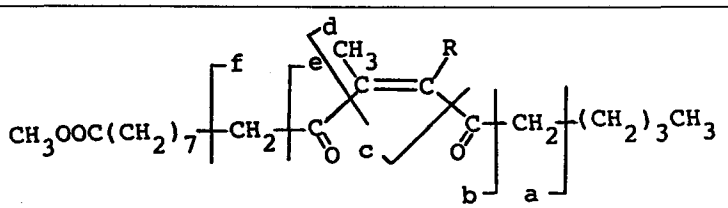


Peak no.	ECL[b]	CCL[c]	m	n	R	Wt. % of total fatty acids	Peak no.	ECL	CCL	m	n	R	Wt. % of total fatty acids
1*	15.09	12	2	4	H	0.02	18*	21.38	19	8	5	H	0.03
2	15.42	12	2	4	CH_3	0.04	19 (F_4)	21.51	18	10	2	CH_3	3.45
3*	17.32	14	4	4	CH_3	trace[d]	20*	21.97	19	8	5	CH_3	trace
4*	17.79	14	6	2	CH_3	trace	21*	22.12	19	9	4	CH_3	0.06
5*	18.33	16	4	6	H	0.09	22*	22.36	19	10	3	CH_3	0.11
6[a]	18.46	16	6	4	H	0.09	23 (F_5)	22.51	20	10	4	H	0.93
7	18.80	16	8	2	H	0.03	24 (F_6)	23.20	20	10	4	CH_3	11.91
8 (F_0)	19.07	16	6	4	CH_3	1.76	25	23.66	20	12	2	CH_3	0.09
9 (F_1)	19.34	16	8	2	CH_3	0.98	26*	23.93	21	10	5	CH_3	0.02
10*	19.56	17	8	3	H	0.02	27*	24.06	21	11	4	CH_3	0.02
11*	19.95	17	6	5	CH_3	0.02	28*	24.40	22	10	6	H	0.04
12*	20.06	17	7	4	CH_3	0.04	29	24.60	22	12	4	H	0.02
13*	20.23	17	8	3	CH_3	0.10	30	25.26	22	12	4	CH_3	0.11
14 (F_2)	20.44	18	8	4	H	1.64	32	24.24		olefinic F_3[e]			0.02
15	20.72	18	10	2	H	0.07	33	24.34					0.02
16*	20.98	18	6	6	CH_3	0.02	34	25.61		olefinic F_6[e]			0.11
17 (F_3)	21.09	18	8	4	CH_3	6.78	35	25.96					0.13

[a]Represents 18.9% of the total lipids of hepatopancreas.
[b]Equivalent chain length.
[c]Carbon chain length.
[d]Less than 0.01%.
[e]F_3 or F_6 methyl ester with one additional double bond, conjugated with a furan ring.
*The acid is unknown.

TABLE 2

Comparison of Major Mass Spectral Fragments of Dimethyldiketo-ene (36) with Those of Monomethyldiketo-ene (40)



Fragments	36 (R=CH_3) m/z (rel. int.)[a]	40 (R=H) m/z (rel. int.)[a]
M^+	352 (48)	338 (9)
$M^+ - H_2O$	334 (68)	320 (20)
$M^+ - OCH_3$	321 (28)	307 (22)
a	295 (55)	281 (3)
a − CH_3OH	263 (100)	249 (10)
b	281 (4)	267 (0)
b − CH_3OH	249 (15)	235 (40)
c	252 (1)	239 (29)
c − CH_3OH	221 (3)	207 (12)
d	153 (4)	139 (23)
e	181 (25)	167 (100)
f	195 (90)	181 (42)
g[b]	177 (29)	163 (18)
h[c]	205 (28)	191 (20)

[a]Relative intensity.
[b]The fragments corresponding to base peaks of olefinic F acids with a double bond in the alkyl chain (Fig. 2B).
[c]The fragments corresponding to base peaks of olefinic F acids with a double bond in the alkylcarboxyl chain (Fig. 2C).

Lipids

Notices
Volume 23, Number 8
August 1988

4th Separation Science and Biotechnology Symposium (August 31–September 3, 1988, Palazzo Feltrinelli, Gargnano del Garda, Italy). Requests for information should be sent to Chairman, Prof. Pier Giorgio Righetti, c/o Euro Business Center, P.O. Box 10552, 1001 EN Amsterdam, The Netherlands.

The 29th International Conference on the Biochemistry of Lipids (September 19–22, 1988, Tokyo, Japan). Sponsored by The Japanese Biochemical Society and the Foundation for Advancement of International Science. For more information, contact Prof. Y. Seyama, Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

Genetic Basis for Obesity and Diabetes (October 30–November 1, 1988, Stouffer Pineisle Resort, Lake Lanier Islands, Georgia). Sponsored by the University of Georgia, Center for Continuing Education and the College of Home Economics. For more information, contact Dr. Carolyn D. Berdanier, Conference Chair, University of Georgia, Department of Foods and Nutrition, Dawson Hall, Athens, GA 30602.

Vitamin E: Biochemistry and Health Implications (October 31–November 3, 1988, The Sheraton Centre, New York, NY). Sponsored by The New York Academy of Sciences. For more information, contact the Conference Department, The New York Academy of Sciences, 2 East 63rd St., New York, NY 10021, (212) 838-0230.

International Symposium on Clinical, Biochemical and Molecular Aspects of Fatty Acid Oxidation (November 6–9, 1988, Penn Tower Hotel, Philadelphia, PA). For more information, contact Paul M. Coates, Ph.D., Division of Genetics, The Children's Hospital of Philadelphia, 34th St. and Civic Center Blvd., Philadelphia, PA 19104.

23rd Annual Hugh Lofland Conference on Arterial Wall Metabolism (May 24–27, 1989, Welches, Oregon). Requests for information should be sent to M. R. Malinow, M.D., Chairman, Hugh Lofland Conference, Oregon Regional Primate Research Center, 505 NW 185th Avenue, Beaverton, OR 97006, (503) 690-5258.

10th Anniversary Meeting of the European Association for Cancer Research (University College, Galway, Ireland, September 11–13, 1989). For further information, contact Dr. S. M. Lavelle, Experimental Medicine, University College, Galway, Ireland; or in North America, contact Dr. J. H. Weisburger, American Health Foundation, Valhalla, New York, NY 10595-1599. The program involves plenary lectures, workshops, symposia and poster sessions.

Dietary Fat Ratios and Liver Plasma Membrane Lipid Composition[1]

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Department of Nutrition, Rutgers University, New Brunswick, NJ 08903

Male Sprague-Dawley weanling rats were fed isocaloric diets consisting of 10% (by wt) fat. The six groups differed in the ratio of corn oil and butter fat present in the diets such that: 10C, 10% corn oil (C); 8C2B, 8% C/2% butter fat (B); 6C4B, 6% C/4% B; 4C6B, 4% C/6% B; 2C8B, 2% C/8% B; and 10B, 10% B. Liver plasma membranes were analyzed for fatty acid composition and cholesterol/phospholipid molar ratio. The 18:2n-6 content was constant in the 10C and 8C2B diets and then decreased linearly through the 2C8B diet. The 20:4n-6 and 18:1n-9 contents were constant except in the 10B diet, in which a significant decrease and increase, respectively, were observed. The cholesterol/phospholipid molar ratio increased between the 10C and 6C4B diets and subsequently (4C6B and 10B diets) remained constant. This data indicates that changes in n-6 fatty acid content in the liver plasma membrane are directly related to dietary intake only for 18:2n-6. Arachidonic acid content in the membrane is maintained at a constant level until the linoleic acid content of the diet is reduced to 0.5% of calories. It also indicates that the cholesterol content of the membrane becomes saturated and does not increase with increasing concentrations of saturated fat in the diet. *Lipids 23,* 829–833 (1988).

It has been proposed in the U.S. that we decrease our total fat consumption from approximately 42% of calories to 30% and alter the ratio of fat intake such that there is an equal distribution of saturated, monounsaturated and polyunsaturated fatty acids (1). However, studies investigating the effects of dietary lipid on membrane lipid structure have generally utilized single-fat sources (2–7) that did not contain an equal ratio of fatty acids. Others have utilized fats that did not contain altered ratios of polyunsaturated to saturated (P/S) fat, but rather monounsaturated/saturated (8) or n-6/n-3 fatty acid alterations (9). In addition, a number of these studies have correlated changes in lipid characteristics with cellular protein function (4,5,7,10,11). These types of dietary fat alterations either demonstrate P/S ratios that differ by several orders of magnitude or monounsaturated/saturated/polyunsaturated (n-6) ratios that are very different from the 1:1:1 recommendations (1). Thus, they are very useful dietary manipulations for determining membrane lipid changes due to extremes of diet. However, the relationship of these single-fat-source diets to a more uniform distribution of classes of fatty acids with respect to membrane lipid composition is not clear.

Investigators have considered the effect of different quantities of individual fatty acids (12,13) or types of fat (14) in the diet on total liver-lipid composition. Those studies were designed to investigate the interrelation of various fats with respect to essential fatty acid deficiency of the whole liver and not a particular membrane's structure or function. Therefore, total liver lipids, as opposed to a particular membrane fraction, were analyzed. We were interested in studying a purified membrane (liver plasma membrane) system that would allow us to examine the changes in membrane lipid composition through a continuum of dietary fatty acid compositions. This allows for a comparison of membrane lipid compositions between the extremes of single-fat-source diets and more intermediate compositions. This will provide a much better understanding of the relationship between the effects of typical experimental diets (single-fat-source) and the recommendations of various national scientific panels regarding dietary fatty acid distribution.

MATERIALS AND METHODS

Forty-eight male Sprague-Dawley weanling rats (Charles River Breeding Labs, Wilmington, MA) were housed individually in wire bottom cages in a temperature-controlled room on a 12-hr light/dark cycle. Animals were divided into six groups and fed an isocaloric diet containing either 10% (w/w) corn oil (10C diet); 8% corn oil/2% butter fat (8C2B diet); 6% corn oil/4% butter fat (6C4B diet); 4% corn oil/6% butter fat (4C6B diet); 2% corn oil/8% butter fat (2C8B diet); or 10% butter fat (10B diet). The compositions of the diets are given in Table 1 and the fatty acid profile in Table 2. The 10B diet contained approximately 220 mg cholesterol/kg diet and the others proportionally less. Diets were formulated by Dr. E. A. Ulman (Research Diets, Inc., New Brunswick, NJ). All diets were fed ad libitum for a period of four wk, at the end of which animals were killed by decapitation. The body wt and liver wt were not different by one-way ANOVA between the groups at the end of the feeding period (data not shown). In several cases, samples were lost so that all animals are not reflected in the final analysis.

Livers were excised and passed through a tissue press. They were then homogenized in three vols of buffer (0.25 M sucrose, 0.5 mM EGTA, 5 mM Hepes, pH 7.4) in a Potter-Elvehjem homogenizer. Plasma membranes were prepared by the Percoll gradient method of Epping and Bygrave (15). Protein was determined by the method of Lowry et al. (16), as modified by Markwell et al. (17). Typical membrane purification with this procedure in our hands is seven- to 20-fold for plasma membrane, 0.5-fold for microsomes and 0.06-fold for mitochondria, as determined by enzyme markers. Plasma membrane protein recovery was 0.3–0.7 mg/gm liver.

Total membrane lipid was extracted with chloroform/methanol (2:1) containing 0.005% BHT, according to the method of Folch et al. (18). Fatty acids were methylated with boron trifluoride according to the procedure of Morrison and Smith (19) and quantitated by gas-liquid capillary chromatography (Hewlett-Packard 5490 gas chromatograph/3392 integrator, SP-2330 30 m capillary column). Individual fatty acids were identified by comparison with known standards. Total membrane cholesterol and phospholipid were determined spectrophotometrically from the Folch extract by the method of Chiamori and Henry (20) and Bartlett (21), respectively.

[1]Presented in part at the FASEB Meeting, Washington, D.C., April, 1987.

*To whom all correspondence should be addressed.

TABLE 1

Diet Composition

Ingredient (gm/kg diet)	10C	8C2B	6C4B	4C6B	2C8B	10B
Casein, alcohol extracted	210	210	210	210	210	210
DL-Methionine	3.2	3.2	3.2	3.2	3.2	3.2
Salt mix[a]	36.8	36.8	36.8	36.8	36.8	36.8
Vitamin mix[b]	10.5	10.5	10.5	10.5	10.5	10.5
Choline bitartrate	2.1	2.1	2.1	2.1	2.1	2.1
Cellulose	52.5	52.5	52.5	52.5	52.5	52.5
Corn starch	543.9	543.9	543.9	543.9	543.9	543.9
Corn oil	91.9	73.5	55.1	36.8	18.4	0
Butter fat	0	18.4	36.8	55.1	73.5	91.9

[a]Salt mix (gm/kg salt mix): Potassium citrate, monohydrate—200; calcium phosphate, dibasic—500; potassium sulfate—52; sodium chloride—74; ferric citrate—6.0; magnesium oxide—24; zinc carbonate—1.6; manganous carbonate—3.5; cupric carbonate—0.3; chromium potassium sulfate—0.55; sodium selenite—0.01; potassium iodate—0.01; sucrose—188.03.

[b]Vitamin mix (gm/kg vitamin mix): Vitamin A palmitate 500,000 IU/gm—0.8; vitamin D_3 400,000 IU/gm—0.25; vitamin E acetate 500 IU/gm—10.0; menadione sodium bisulfite—0.08; biotin 1.0%—2.0; cyanocobalamin 0.1%—1.0; folic acid—0.2; niacin—3.0; calcium pantothenate—1.6; pyridoxine HCl—0.7; riboflavin—0.6; thiamin HCl—0.6; sucrose—979.17.

TABLE 2

Diet Fatty Acid Composition (Wt %)

Fatty acid	10C	8C2B	6C4B	4C6B	2C8B	10B
<C12:0	0	0.6	1.1	1.7	2.3	2.2
C12:0	0	0.9	1.7	2.6	3.4	3.7
C14:0	0	2.8	5.5	8.5	11.0	12.6
C14:1	0	0.3	0.6	1.0	1.3	1.5
C16:0	10.7	15.0	19.5	24.1	28.6	33.3
C16:1	0.2	0.3	0.6	0.9	1.2	2.2
C18:0	1.5	3.7	6.0	8.3	10.8	12.8
C18:1	25.2	25.1	25.9	26.4	27.6	28.2
C18:2	62.4	51.3	39.1	26.4	13.9	3.4
P/S ratio[a]	5.1	2.2	1.2	0.6	0.2	0.05
P/M/S[b]	62/25/12	51/26/23	39/27/34	26/28/45	14/30/56	3/32/65
n-6 Fatty acids (% cal)[c]	12.1	10.0	7.5	5.4	2.6	0.4

[a]Polyunsaturated fatty acid/saturated fatty acid ratio in diet.

[b]Ratio (wt %) of polyunsaturated/monounsaturated/saturated fatty acids.

[c]Percentage of calories as n-6 essential fatty acids.

TABLE 3

Weight Percent Fatty Acid Composition of Liver Plasma Membranes[a]

	Diet					
Fatty acid	10C (7)	8C2B (7)	6C4B (7)	4C6B (8)	2C8B (8)	10B (7)
16:0	24.2 ± 1.8[a]	24.7 ± 1.2[a]	24.6 ± 1.3[a]	24.0 ± 1.3[a]	24.0 ± 3.5[a]	27.5 ± 1.4[a]
16:1 n-7	1.7 ± 0.4[a]	1.6 ± 0.2[a]	1.8 ± 0.2[a,c]	2.1 ± 0.3[a,c]	3.0 ± 0.6[c,d]	3.5 ± 0.5[d]
18:0	16.7 ± 0.7[a]	16.7 ± 0.5[a]	16.9 ± 0.9[a]	18.5 ± 1.0[a]	18.3 ± 1.2[a]	19.0 ± 0.9[a]
18:1 n-9	15.0 ± 1.0[a]	14.3 ± 1.0[a]	17.7 ± 1.2[a]	15.0 ± 1.1[a]	17.1 ± 1.4[a]	22.3 ± 1.6[b]
18:2 n-6	15.6 ± 0.9[a,b]	16.0 ± 0.5[a]	13.6 ± 0.8[b]	11.3 ± 0.9[c]	9.1 ± 0.7[d]	5.0 ± 0.1[e]
20:4 n-6	19.3 ± 1.3[a]	20.0 ± 0.7[a]	18.3 ± 1.2[a]	19.9 ± 1.5[a]	18.8 ± 2.0[a]	12.9 ± 1.3[b]
22:5 n-3	1.9 ± 0.3[a]	1.8 ± 0.3[a]	1.0 ± 0.3[a,b]	1.6 ± 0.3[a]	0.4 ± 0.2[b]	0.3 ± 0.2[b]
22:6 n-3	2.1 ± 0.2[a]	2.4 ± 0.1[a,b]	2.2 ± 0.2[a,b]	3.3 ± 0.2[b,c]	3.6 ± 0.5[c]	5.4 ± 0.7[d]
Total n-6	34.9 ± 2.0[a]	35.9 ± 0.4[a]	31.9 ± 1.6[a,b]	31.2 ± 2.2[a,b]	27.9 ± 2.3[b]	17.9 ± 1.3[c]
Total n-3	4.0 ± 0.5[a,b]	4.2 ± 0.4[a,b]	3.3 ± 0.5[a]	5.0 ± 0.4[b,c]	4.1 ± 0.6[a,b]	5.8 ± 0.7[c]
n-6 Fatty acids in diet (% cal)	12.1	10.0	7.5	5.4	2.6	0.4

[a]Data are mean ± SEM for the number of animals indicated below each dietary group. Values in a row with common superscripts are not statistically different than one another. All other values in a row are different at $p < 0.05$.

Data were analyzed for variance and by Duncan's multiple range test, utilizing the SAS Statistics package, or regression analysis where appropriate.

RESULTS

The fatty acid profiles of liver plasma membranes from animals fed the various diets are presented in Table 3. There were no changes observed in the saturated fatty acids between any of the dietary groups, with 16:0 comprising approximately 24% and 18:0, 17–19% of the total fatty acid. There were, however, alterations in the various unsaturated fatty acids. 16:1n-7 remained constant in liver plasma membranes from the 10C- through the 4C6B-fed animals, but then increased significantly in those from the 2C8B- and 10B-fed animals. A similar change was observed with 18:1n-9. It was constant in liver plasma membranes isolated from animals fed the 10C through the 2C8B diets, but increased significantly in the 10B-diet-fed animals. In contrast to these modest changes observed in the monounsaturated fatty acids, there were marked changes in the polyunsaturated fatty acids as the ratio of fat in the diet was altered. This was observed most clearly for 18:2n-6. There was a decline that was not apparent (Table 3) until the 6C4B diet. That is, liver plasma membranes prepared from animals fed the 10C and 8C2B diets were not significantly different than one another, but there was a steady decline in the percentage of 18:2n-6 with the remainder of the groups. This can also be observed in Figure 1 as a linear decline in the [18:2n-6/(16:1n-7 + 18:1n-9)] ratio, as the percentage of corn oil in the diet was decreased ($r = 0.816$, $p < 0.001$).

This linear decrease is not seen in the case of arachidonic acid (20:4n-6). Arachidonic acid in the liver plasma membrane remained at a constant level (approximately 19%) until all of the corn oil had been eliminated from the diet (10B diet). At this point it declined to 12.9%. When the data were replotted as the ratio of 20:4n-6/18:2n-6 (Figure 2), it is evident that the ratio was constant in liver plasma membranes from the 10C- through 6C4B-fed animals (100–60% of fat source as corn oil) and then increased linearly. Furthermore, the total n-6 fatty acids in liver plasma membranes remained constant (diets 10C through 4C6B), up to the point where n-6 fatty acids comprised less than 3% of the calories (2C8B and 10B diets).

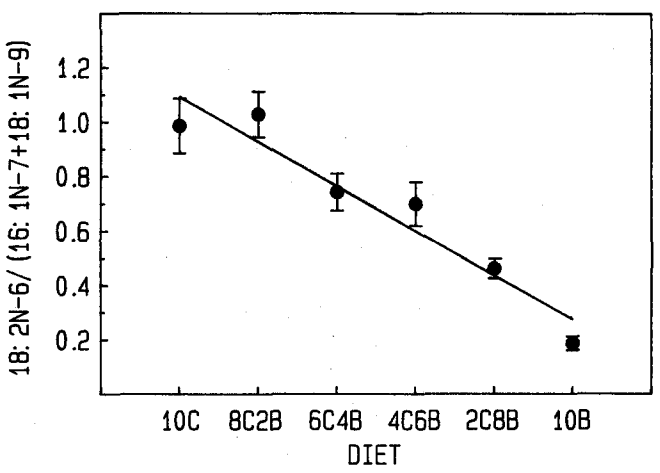

FIG. 1. 18:2n-6/[16n-7 + 18:1n-9] Fatty acid ratio vs diet. All data are X ± SEM. The line was fitted by linear regression to: Y = (−0.816)X + 1.259 with r = 0.816 (p < 0.05). By ANOVA values for diets 10C and 8C2B were significantly greater than all others. Diets 6C4B, 4C6B and 2C8B were significantly different than 10C, 8C2B and 10B, while diet 10B was different than all others. Values were considered significant when p < 0.05.

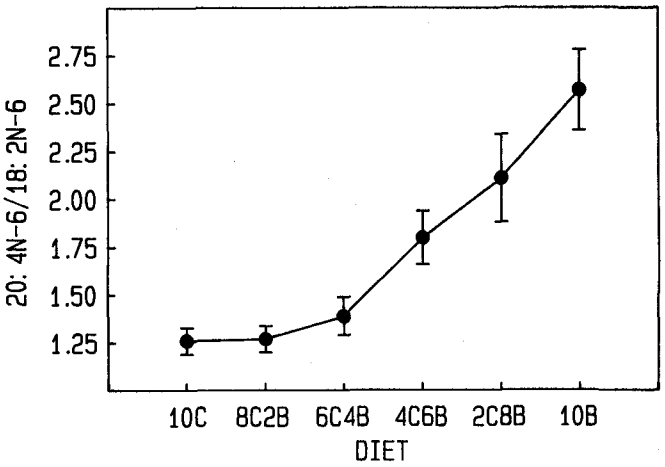

FIG. 2. 20:4n-6/18:2n-6 Fatty acid ratio vs diet. All data are X ± SEM. Statistical differences were as follows. Different letters associated with each diet indicate that values are significantly different. Diet 10C, a; 8C2B, a; 6C4B, a,b; 4C6B, b,c; 2C8B, c; 10B, d. Values were considered significant when p < 0.05.

The liver plasma membrane content of 22:5n-3 decreased, while that of 22:6n-3 increased, as butter fat was added to the diets. The total of n-3 fatty acids in the membrane remained relatively constant.

The cholesterol and phospholipid values of the liver plasma membranes from animals fed the various diets are presented in Table 4. There was an increase (although not statistically significant) in the cholesterol/phospholipid molar ratio of the membranes as the butter fat content of the diet was increased from 10C to 6C4B, which remained relatively constant for diets 4C6B and 10B. The 2C8B diet (0.475 ± 0.057) was anomalous in this regard. The cholesterol/phospholipid molar ratio was significantly lower in liver plasma membranes from the 10C- and 2C8B-fed animals compared with the 4C6B- and 10B-fed animals ($p < 0.05$). The cholesterol and phospholipid levels relative to protein content were not different between any of the groups.

DISCUSSION

There have been a number of investigations through the years that have studied the effects of dietary lipids on membrane structure (2–9,22). However, the vast majority of these have investigated single-fat-source diets as opposed to ratios of the dietary fats. This distorts the dietary lipid composition markedly towards highly saturated, monounsaturated or polyunsaturated fatty acid diets. For example, in the present study, the 10C diet is composed of 62% polyunsaturated, while the 10B diet is composed of 65% saturated fatty acids. This study demonstrates that an approach of utilizing ratios of fats producing intermediate fatty acid compositions may be an important consideration in determining the effect of

TABLE 4

Cholesterol and Phospholipid Composition of Liver Plasma Membranes[a]

Diet	Cholesterol μmol/mg protein	Phospholipid μmol/mg protein	Cholesterol/Phospholipid molar ratio
10C (5)	0.096 ± 0.014[a]	0.203 ± 0.040[a]	0.485 ± 0.036[a]
8C2B (7)	0.108 ± 0.019[a]	0.199 ± 0.037[a]	0.569 ± 0.072[a,b]
6C4B (6)	0.120 ± 0.020[a]	0.187 ± 0.028[a]	0.643 ± 0.081[a,b]
4C6B (7)	0.126 ± 0.028[a]	0.202 ± 0.047[a]	0.646 ± 0.049[a,b]
2C8B (4)	0.126 ± 0.020[a]	0.284 ± 0.064[a]	0.475 ± 0.057[a]
10B (6)	0.104 ± 0.014[a]	0.155 ± 0.026[a]	0.697 ± 0.057[b]

[a]Data are mean ± SEM for the number of animals indicated for each dietary group. Values in a column with common superscripts are not statistically different than one another at $p < 0.05$.

dietary fat on biochemical and physiological function. The data in the present study illustrate that single sources of dietary fat produce liver plasma membrane changes that are not necessarily representative of those produced by intermediate ratios.

The most useful comparison would relate the 6C4B diets with the 10C and 10B diets. The 6C4B diet had a polyunsaturated/monounsaturated/saturated fatty acid ratio of approximately 1:1:1 (Table 2). This is the fatty acid ratio currently recommended by a variety of organizations (1) relative to optimal human consumption patterns.

Liver plasma membranes from animals fed the 6C4B diet exhibited a cholesterol/phospholipid molar ratio most similar to those from animals fed the 10B diet (Table 4) and a fatty acid profile more similar to those from animals fed the 10C diet. In other studies utilizing single-fat sources, we have found that the major changes observed between butter and corn oil are a shift in the 18:2n6/(18:1n-9 + 16:1n-7) ratio (23). Thus, we have chosen to compare the ratio of these two fatty acids across the diets. The slight decline in 18:2n-6 from animals fed the 6C4B diet, relative to the 10C diet, was not statistically significant (Table 3), but is clearly seen with linear regression when expressed as a ratio with n-9 and n-7 unsaturated fatty acids (Fig. 1) ($p < 0.05$). Furthermore, a slight shift in the ratio (4C6B diet) produces a significant change in the amount of 18:2n-6 (Table 3) and the ratio of 20:4n-6/18:2n-6 (Fig. 2) found in the liver plasma membrane. However, the 18:2n-6/(18:1n-9 + 16:1n-7) ratio was not different between these two groups.

Thus, the use of single-fat-source diets, while being extremely useful in determining the effects of extremes in dietary modifications on changes in cell structure and function, may not be precise enough to predict changes to be expected within the framework of human nutritional modifications.

It is also of interest to compare this data with that of Caster et al. (14), relating the intake of various dietary lipids to total liver lipid composition. They reported no correlation between the saturated fatty acid content of the diet and the saturated fatty acid content of total liver lipid, when studied with an array of common fatty acids in various ratios. This is a generally observed phenomenon, indicating that total organ lipid saturated fatty acids are very refractory to change, and it is consistent with our data. We also observed similar effects in the case of the monounsaturates, primarily 18:1n-9. The animals maintained a fairly constant amount of monounsaturates in the liver plasma membrane (Table 3). This is in relatively good agreement with the work of Caster et al. (7), in which a low correlation between the 18:1n-9 dietary- and tissue-total lipid concentration was reported. We consider these compensatory changes by the organism an attempt to balance the decline seen in the n-6 polyunsaturates as large amounts of saturated fat is added to the diet.

The major change seen in these membranes was in the amount of 18:2n-6. As seen in Table 3, the wt% of 18:2n-6 in liver plasma membranes remained constant with the first two diets, which supplied 100% and 80% of the fat source as corn oil. A linear decline was observed through the rest of the diets, as the percentage of calories from linoleic acid went below 10%. It is interesting to note from Table 2 that the amount of arachidonic acid remained constant in the plasma-membrane total fatty acids as long as the percentage of calories from 18:2n-6 was greater than 0.5% (of calories). This becomes interesting in light of the current recommendations that 1% of our calories be derived from linoleic acid. There is no change in rats, with respect to wt gain or dermal score (2), as the linoleic acid content of the diet is increased above 1% of calories. This may be interpreted as arachidonic acid changes being the primary determinant of n-6 deficiency symptoms, because membrane linoleic acid concentration decreased at dietary concentrations well in excess of reported requirements. It should be noted, however, that we did not observe significant amounts of 20:3n-9, a biochemical marker for essential fatty acid deficiency (25), or any visual signs of deficiency in these animals. This is not entirely surprising, because the animals were only maintained on these diets for four wk.

A recent report by Nasser et al. (9) investigated the effect of various ratios of n-3/n-6 dietary lipids on total liver-phospholipid fatty acid composition. No changes were reported in 18:2n-6 content over a range of evening primrose oil (high 18:2n-6 and 18:3n-6) and polepa (n-3 rich marine fish oil) ratios. Their lowest percent of evening primrose oil was 50% of the total fat content, an amount similar to those amounts in the 6C4B and 4C6B diets in the present study. At this point, however, we observed significant decreases in 18:2n-6, while they observed no

such differences. This difference in the two studies could be due to the different types of oil mixtures or differences in the samples. In the present study, total fatty acid profile of purified liver plasma membranes was analyzed, while in the study of Nasser et al. (9), the total liver-phospholipid fatty acid profile was determined.

The cholesterol/phospholipid molar ratio exhibited an increase commensurate with the quantity of cholesterol and saturated fat in the diet up to a saturation point (6C4B diet). The exception to this was liver plasma membranes from animals fed the 2C8B diet. The marked change at this dietary fat ratio is unclear. It should be noted that this decrease was due primarily to an increase in the phospholipid/mg protein content.

It is clear from this study that single-fat sources do not necessarily give a true indication of the purified membrane lipid composition of mixed fats. This is especially important in studies attempting to relate changes in function of membrane proteins with dietary fat alteration. The range of modification that most individuals will be able to make in their dietary pattern is much more limited than the single-fat-source feeding studies most often performed in rats. This is not meant to imply that such studies are not useful, but rather to point out that, if animal studies are to be used as an aid in determining potential alterations in cell function by dietary lipid in human populations, more work must be addressed at intermediate ratios of these fats. For example, if the 6C4B were taken as approximately a 1:1:1 ratio of polyunsaturated/monounsaturated/saturated fatty acids, the fatty acid composition would be similar to the 10C diet, whereas the cholesterol/phospholipid molar ratio would be most similar to the 10B diet. Both of these components could have an effect on membrane protein function and hence, it is important to investigate the interaction of these changes.

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REFERENCES

1. *Recommended Dietary Allowances,* 9th revised edn., pp. 31-38, Committee on Dietary Allowances, Food and Nutrition Board, National Academy of Sciences, Washington, DC.
2. Clandinin, M.T., Field, C.J., Hargreaves, K., Morson, L., and Zsigmond, E. (1985) *Can. J. Physiol. Pharmacol. 63,* 546-556.
3. Kasofsky, J.M., and Hamm, M.W. (1986) *Fed. Proc. 45,* 354 (Abstract).
4. Neelands, P.J., and Clandinin, M.T. (1983) *Biochem. J. 212,* 573-583.
5. Galli, C., Agradi, E., Petroni, A., and Tremoli, E. (1981) *Lipids 16,* 165-172.
6. Tahin, Q.S., Blum, M., and Carafoli, E. (1981) *Eur. J. Biochem. 121,* 5-13.
7. Stubbs, C.D., and Smith, A.D. (1984) *Biochim. Biophys. Acta 779,* 89-137.
8. Kramer, J.K.G. (1980) *Lipids 15,* 651-660.
9. Nasser, B.A., Huang, Y.S., Manku, M.S., Das, U.N., Morse, N., and Horrobin, D.F. (1986) *Lipids 21,* 652-656.
10. Kasofsky, J., and Hamm, M.W. (1987) *Fed. Proc. 46,* 1476 (Abstract).
11. Spector, A.A., and Yorek, M.A. (1985) *J. Lipid Res. 26,* 1015-1035.
12. Holman, R.T. (1986) in *Dietary Fat and Cancer* (Ip, C., Birt, D.F., Rogers, A.E., and Mettlin, C., eds.), pp. 211-228, Alan R. Liss, New York, NY.
13. Mohrhauer, H., and Holman, R.T. (1963) *J. Lipid Res. 4,* 151-159.
14. Caster, W.O., Mohrhauer, H., and Holman, R.T. (1966) *J. Nutr. 89,* 217-225.
15. Epping, R.J., and Bygrave, F.L. (1984) *Biochem. J. 223,* 733-745.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
17. Markwell, M.A.K., Hass, S.M., Bieber, L.L., and Tolbert, N.E. (1978) *Anal. Biochem. 87,* 206-210.
18. Folch, J., Lees, M., and Stanley, G.H.S. (1957) *J. Biol. Chem. 226,* 497-509.
19. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600-608.
20. Chiamori, N., and Henry, R.J. (1959) *Am. J. Clin. Pathol. 31,* 305-309.
21. Bartlett, G.R. (1959) *J. Biol. Chem. 234,* 466-468.
22. Morson, L.A., and Clandinin, M.T. (1985) *Nutr. Res. 5,* 1113-1120.
23. Lee, C-R., and Hamm, M.W. (1988) *FASEB J. 2,* A640.
24. Gibson, R.A., McMurchie, E.J., Chernock, J.S., and Kneebone, G.M. (1984) *Lipids 19,* 942-951.
25. Brenner, R.R. (1981) *Prog. Lipid Res. 20,* 41-47.

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Comparison of Free α-Tocopherol and α-Tocopheryl Acetate as Sources of Vitamin E in Rats and Humans

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The uptake of α-tocopherol from 2*R*,4′*R*,8′*R*-α-tocopherol and 2*R*,4′*R*,8′*R*-α-tocopheryl acetate has been compared in rats and humans. The two forms of vitamin E were compared simultaneously in each subject (rat and human) by using a combination of deuterium-substitution and gas chromatography–mass spectrometry (GC–MS) to distinguish and measure the competitive uptake of α-tocopherol from an orally ingested mixture of the acetate and the free phenol forms. When rats were dosed in a manner analogous to that used in traditional bioassays, i.e., providing the two forms of vitamin E once daily in tocopherol-stripped corn oil for four successive days immediately prior to sacrifice, the net uptake of α-tocopherol from the free phenol form was only half that from the acetate. This result is consistent with the greater activity of the acetate that had been observed previously in bioassays. However, when the two forms of tocopherol were intubated into rats as a single dose mixed in with an aqueous bolus of standard laboratory diet, the amount of α-tocopherol taken up from the free form after 24 hr was very similar to that derived from the acetate. In five adult humans, competitive uptake studies of the two forms after a single dose taken with a meal showed that the amount of α-tocopherol from the free phenol form was equal to that from the acetate in plasma and red blood cells. These findings illustrate the value and potential of using deuterium-substituted α-tocopherol and GC–MS in evaluating the effectiveness of different forms of vitamin E in human studies. The results also stress the need for caution in using data obtained from animal bioassays when considering comparative human nutritional standards.

Lipids 23, **834–840 (1988).**

α-Tocopheryl acetate (α-T-Ac) is the most common form of vitamin E used for oral supplementation in humans. In the gut, the acetate is hydrolyzed with the aid of pancreatic enzymes and bile to yield free α-tocopherol (α-T-OH) which is then partially absorbed through the intestinal wall into the lymph (1–3). No measurable α-T-Ac is found in blood serum (4). Naturally occurring vitamin E is present in food as the free phenol.

The relative bioavailabilities of α-T-OH and α-T-Ac have been of interest for many years. In 1949, Harris and Ludwig (5) reported the surprising result that *dl*-α-T-OH was only about 0.6 times as active as *dl*-α-T-Ac in the rat fetal resorption assay. (It is almost certain that at the time this study was conducted the *dl*-form was synthesized using natural phytol and was therefore a mixture of equal amounts of the 2*R*,4′*R*,8′*R*- and 2*S*,4′*R*,8′*R*-diastereoisomers [1]. The mixture of these two α-tocopherols is now designated as *ambo*-α-tocopherol.) Harris and Ludwig suggested that the lower activity of the nonesterified form was the result of a diet-mediated oxidative destruction of the compound in the gut. In support of this, they reported that simultaneous feeding of the antioxidant, diamylhydroquinone, with free α-tocopherol significantly increased the effectiveness of the tocopherol in the bioassay (5). Much later, Leth and Sondergaard (6) confirmed the lower activity of the nonesterified form, reporting that, in this case, *all-rac*-α-T-OH (i.e., a mixture of equal amounts of the eight possible diastereoisomers) was less active than *all-rac*-α-T-Ac in both the fetal resorption and red cell hemolysis assays, by factors of 0.79 and 0.85, respectively. (The authors did not indicate whether the relative potencies were calculated on a weight or a molar basis.) They also reported, however, the surprising result that the two forms of vitamin E exhibited identical activities in the liver storage assay. Further confirmation of the lower potency of α-T-OH was recently provided by Wieser and coworkers (7), who reported results obtained using the fetal resorption, plasma pyruvate kinase (myopathy) and liver storage assays. On a molar basis, it was found that *all-rac*-α-T-OH was less active than *all-rac*-α-T-Ac in all three assays by factors of 0.47, 0.57 and 0.62, respectively.

On the basis of the findings from these animal tests, it has been suggested (6) or implied (7) that the biopotencies of *all-rac*-α-T-OH and *RRR*-α-T-OH, expressed relative to *all-rac*-α-T-Ac as the standard, should be lowered from their officially accepted values (8) of 1.10 and 1.49, respectively. (The difference between the accepted biopotencies of the acetate and the corresponding free tocopherol arises solely from the difference in the molecular weights of the two forms. The officially accepted biopotency of *RRR*-α-T-Ac relative to *all-rac*-α-T-Ac is 1.36 [8].)

In humans, studies have been limited to comparing the absolute increases in the level of plasma α-T-OH in individuals administered large, single, oral doses of either the free tocopherol or the acetate. The results of these studies have suggested that, in contrast to rats, the free form is slightly more potent than the acetate.

Thus, in 1952, Week and coworkers (9) reported higher plasma levels of α-T-OH in subjects given *all-rac*-α-T-OH compared with subjects given an equimolar amount of *all-rac*-α-T-Ac. Much later, Baker and coworkers (10) appeared to confirm this finding in subjects given 400–1600 mg of either *all-rac*-α-T-OH or its acetate in both short-term (24 hr) and long-term (21 days) studies. In this investigation, equal weights of the free phenol and of the acetate were compared. It is not evident from the data, however, that the increases in plasma tocopherol levels in the subjects who took the free phenol, compared with the data from those who took the acetate, were significantly greater than the ca. 10% that would have been

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Abbreviations: α-T-Ac, α-tocopheryl acetate; α-T-OH, α-tocopherol; EDTA, ethylenediamine tetraacetic acid; d_3-*RRR*-α-T-OH, 2*R*,4′*R*,8′*R*-α-(5-C^2H_3)tocopherol; d_6-*RRR*-α-T-OH, 2*R*,4′*R*,8′*R*-α-(5,7-$(C^2H_3)_2$)tocopherol; d_9-α-T-OH, *ambo*-α-(5,7,8-$(C^2H_3)_3$)tocopherol; SDS, sodium dodecyl sulfate; GC–MS, gas chromatography–mass spectrometry; HPLC, high performance liquid chromatography; RBC, red blood cells.

expected simply from the difference in molecular weight between α-T-OH and α-T-Ac. More recently, Horwitt and coworkers (11) have reported that subjects given 800 IU of *RRR*-α-T-OH showed a mean increase of 71% in their serum levels of α-T-OH after 24 hr, compared with a smaller increase of 61% in subjects given 800 IU of *RRR*-α-T-Ac (The doses were equimolar, in conformity with the existing USP standard.).

Recently, we have used deuterium-substituted α-tocopherols and gas chromatography–mass spectrometry (GC–MS) to probe the biokinetics of the uptake of dietary α-tocopherol into rat tissues (12). In this paper, we report results obtained by applying this technique directly to the question of the relative uptake, in rat and man, of α-T-OH derived from the free tocopherol and acetate forms of vitamin E. In contrast to the experimental methods used earlier by others, we have taken advantage of the fact that, by distinguishing the free tocopherol and acetate with tri- or hexadeutero-substitution (i.e., differential labeling), it is possible to measure the competitive uptake of the two forms, when given as a mixture of known composition to a single subject. This approach has the advantage of eliminating the effects of variations in absolute uptake between individual subjects. Individual variation in net uptake is an important factor confounding the interpretation of the results of studies obtained with conventional techniques using nonlabeled compounds. Only one or the other of the two forms being compared can be tested at one time on each individual, which requires the use of a large number of subjects in order to obtain statistically significant results.

Another advantage of our method is that the deuterated α-tocopherol absorbed from the mixture is easily distinguished from the nondeuterated, background α-tocopherol already present in the subject.

EXPERIMENTAL

Synthesis of deuterated RRR-*α-tocopherols. RRR*-α-(5-C^2H_3)tocopherol (d_3-*RRR*-α-T-OH) and *RRR*-α-(5,7-(C^2H_3)$_2$)tocopherol (d_6-*RRR*-α-T-OH) were synthesized, as described previously (12,13), by deuteriomethylation of *RRR*-γ- and *RRR*-δ-tocopherols, respectively. The γ- and δ-tocopherols were obtained from soybean oil concentrate. The tocopheryl acetates were prepared as described previously (12). While this work was in progress, *ambo*-α-(5,7,8-(C^2H_3)$_3$)tocopherol (d_9-α-T-OH) was prepared and was used as an internal standard for quantitating d_0-, d_3- and d_6-α-T-OH in some of the experiments. The details of the synthesis of this compound will be reported elsewhere.

The degree of incorporation of the nominal amount of deuterium into d_3-*RRR*-α-T, d_6-*RRR*-α-T and d_9-α-T (acetate or free tocopherol) ranged from 75% to 90%, as determined by GC–MS. The remaining material was composed principally of α-tocopherol, containing one less deuterium atom and, to a much lesser extent, two less deuterium atoms. All measurements of deuterated α-tocopherols were corrected accordingly, to reflect the true amount of the particular α-tocopherol present in each sample.

Measurement of α-T-OH by GC–MS. α-T-OH was extracted from plasma by the ethanol/heptane method and from tissues, red blood cells, intestinal and fecal material by the sodium dodecyl sulfate (SDS) method (12,14). The heptane lipid extract was passed through an analytical, high performance liquid chromatography (HPLC) silica gel column and the recovered α-T-OH fraction was trimethylsilylated after removal of the HPLC eluent by evaporation under a stream of nitrogen (12). When it became available, d_9-α-T-OH was added as an internal standard (ca. 6 nmol) in decane solution immediately prior to extraction of the sample.

The tocopheryl trimethylsilyl ethers were injected into a 12 m × 0.2 mm ID Ultra-1 (OV 101 methyl silicone) capillary column in a Hewlett-Packard benchtop model 5995 GC–MS, operating in the single-ion monitoring mode (12). The 502 (d_0-α-T), 505 (d_3-α-T), 508 (d_6-α-T) and 511 (d_9-α-T) parent trimethylsilyl ether molecular ions were monitored continuously and their corresponding peak areas integrated to give the relative abundance of each tocopherol.

GC–MS also was used to confirm the composition of the mixture of deuterated α-tocopherols provided to the animal or human subjects in each experiment. In those experiments in which the starting molar ratio was significantly different from unity, the ratios found in the tissues and blood were corrected accordingly.

Rat studies: Experiment 1. Two pairs of ten-week-old male Sprague-Dawley rats (ca. 300 g) were dosed daily for four days with an equimolar mixture of d_3-*RRR*-α-T-Ac and d_6-*RRR*-α-T-OH (combined dose: 2 mg/kg body wt/day) dissolved in ca. 100 μl of tocopherol-stripped corn oil that had no added antioxidant. One pair of rats was maintained on a vitamin E-free AIN-76A diet (15) and the other on a vitamin E-sufficient AIN-76A diet (50 mg *all-rac*-α-T-Ac/kg diet) from weaning (3 wk) through the duration of the experiment. (The proportion of corn oil in each diet was increased from 5% to 10% with a compensating decrease in the proportion of corn starch.) The food was provided ad libitum. Blood and tissues were obtained on the day after the last dose. The blood was obtained by heart puncture under halothane anesthesia, and the tissues from the anesthetized animals were then dissected. All of the fecal material in the large intestine was collected and care was taken to ensure that the total combined sample from each animal was analyzed. All samples were stored at −80 C prior to analysis.

Experiment 2. Five 13-week-old, nonfasted male Sprague-Dawley rats weighing 425–510 g were intubated with about 5 ml of an aqueous slurry of AIN-76A diet (5% corn oil; 20 g diet slurried in 15 ml water) containing ca. 3.8 g of dry mixture and 4 mg of a mixture of d_3-*RRR*-α-T-Ac and d_6-*RRR*-α-T-OH. The weighed deuterated tocopherols were first dissolved in the correct amount of tocopherol-stripped corn oil (with no added antioxidant) and were then thoroughly mixed with the dry base diet (no corn oil) before adding water to make a slurry. This was done immediately prior to dosing. After dosing, the rats were returned to their cages and permitted uninterrupted access to water and their usual AIN-76A diet. Blood was obtained as described above at four and 24 hr after dosing. Tissues were obtained at 24 hr by the procedure already described for Experiment 1. In addition, the small intestine was divided into three equal-sized segments and each segment, including its contents, was subsequently analyzed for α-tocopherol.

Human studies: Experiment 3. Trials were performed on five healthy adults (three males and two females, ages

36-59 years), who were not taking any form of medication. A neat, equimolar mixture of d_3-*RRR*-α-T-OH and d_6-*RRR*-α-T-Ac was weighed out into gelatin slip-joint capsules (ca. 100 mg total/capsule). The capsules were always taken with an evening meal.

Part A: A total of two capsules, one per day, was taken by each of two males (GB and KI) on successive days. Blood, collected over disodium EDTA, was obtained by venipuncture from both subjects ca. 15 hr after taking each capsule and, for one of the subjects (KI), two days after the last capsule. The ratio of the two deuterated α-T-OH's was determined in the lipid extracts obtained from plasma and red blood cells in the usual way (12).

Part B: Two females (HB and SL) and one male (DF) each took one capsule, only, of 100 mg of the mixture of deuterium-substituted tocopherols. Blood was obtained from the subjects ca. 19 and 43 hr after taking the capsule.

RESULTS

The data for the competitive uptake into rat blood and tissues of α-tocopherol from a mixture of free α-tocopherol and α-tocopheryl acetate are presented as OH/Ac ratios in Tables 1 (Experiment 1) and 2 (Experiment 2).

The combined mean value of the OH/Ac ratio for the four animals dosed daily for four successive days with the corn oil mixture (Experiment 1) was found to be 0.49 ± 0.05 (SEM). Clearly, in this experiment, α-tocopheryl acetate was about twice as effective a source of absorbed α-T-OH. The combined amounts of absorbed deuterated

TABLE 1

Ratios of d_6-α-T-OH/d_3-α-T-OH Showing the Competitive, Net Uptake of α-T-OH Derived from Free and Esterified α-Tocopherols[a]

Diet	E−		E+	
Rat	1	2	3	4
Liver	0.50	0.48	0.41	0.38
RBC	0.48	0.50	0.59	0.45
Plasma	0.49	0.55	—	0.62
Spleen	0.50	0.43	0.67	0.41
Kidney	0.44	0.39	0.53	0.41
Lung	0.48	0.43	0.74	0.51
Heart	0.58	0.58	0.67	0.55
Muscle	0.50	0.49	—	0.30
Testes	0.55	0.58	0.33	0.30
Brain	0.46	0.41	0.53	0.33
Feces[b]	—	0.55	0.90	0.91
Mean[c]	0.50	0.48	0.56	0.43
(±SD)	±0.04	±0.07	±0.14	±0.11

[a]I.e., OH/Ac, after four daily doses (2 mg/kg/day) of an equimolar mixture of d_6-α-T-OH and d_3-α-T-Ac given to rats maintained on either a vitamin E-deficient (E−) or a vitamin E-sufficient (E+) diet (Experiment 1).

[b]Does not include recovered α-T-Ac. Recoveries of acetate (free tocopherol in parentheses) were 5.8 (88.9), 1.5 (95.1) and 7.0 (97.2) nmol/g of feces in rats 2, 3 and 4, respectively.

[c]Excluding feces.

TABLE 2

Competitive Net Uptake of α-Tocopherol, OH/Ac, and Percent Total Deuterated α-Tocopherol Relative to Total α-Tocopherol in Tissues and Blood of Five Rats[a]

					Small intestine[b]				Plasma		RBC	
Rat	Liver	Spleen	Lung	Heart	1	2	3	Feces	4 hr	24 hr	4 hr	24 hr
OH/Ac (d_6-*RRR*-α-T-OH/d_3-*RRR*-α-T-OH):												
1	0.97	1.00	1.03	1.01	0.96	0.91	0.77	0.94	1.05	0.99	1.13	1.01
2	1.00	1.03	1.01	1.04	1.05	0.99	1.00	1.05	1.05	0.97	1.33	1.04
3	0.97	0.99	1.02	1.09	1.03	0.98	1.03	1.02	1.05	0.94	0.99	0.98
4	0.95	1.01	1.16	1.65	0.96	0.81	0.92	1.09	0.96	0.90	1.23	1.01
5	0.97	1.02	1.26	1.61	1.12	0.95	—	1.01	1.00	0.97	1.03	0.99
Mean:	0.97	1.01	1.10	1.28	1.03	0.93	0.93	1.02	1.02	0.96	1.14	1.01
S.D.	0.02	0.02	0.11	0.32	0.07	0.07	0.12	0.05	0.04	0.04	0.14	0.02
Percent total deuterated α-tocopherol[c]:												
1	44.3	47.0	37.3	11.5	44.9	54.8	50.6	74.6	58.1	52.0	24.4	53.8
2	51.9	48.9	37.6	10.5	44.8	43.7	23.8	76.5	44.2	56.2	22.3	59.0
3	45.7	50.1	37.2	14.0	36.7	45.1	45.7	83.7	39.3	47.4	16.9	57.6
4	49.1	48.2	35.3	13.8	39.7	53.9	36.9	83.7	56.2	55.1	22.5	35.6
5	54.6	47.2	43.5	14.2	43.1	48.4	—	60.1	59.4	55.6	23.5	58.8
Mean:	49.1	48.3	38.2	12.8	41.8	49.2	39.2	75.7	51.5	53.3	21.9	53.0
S.D.	4.3	1.2	3.1	1.7	3.5	5.0	11.7	9.6	9.1	3.7	2.9	9.9

[a]Rats were fed a single dose of a mixture of d_3-*RRR*-α-T-Ac and d_6-*RRR*-α-T-OH mixed in an aqueous bolus of laboratory diet (Experiment 2). Results corrected for an initial d_6-*RRR*-α-T-OH/d_3-*RRR*-α-T-Ac mole ratio = 1.372.

[b]The small intestine (including contents) was divided into three equal segments starting at the end closest to the stomach. The free α-T-OH was separated from α-T-Ac by HPLC. The data in the table refer only to the free α-T-OH.

[c]100 × (d_3-α-T-OH + d_6-α-T-OH)/(d_0-α-T-OH + d_3-α-T-OH + d_6-α-T-OH).

α-tocopherols, expressed as a percentage of the total α-tocopherol in each of the tissues and blood fractions examined, are compared for the vitamin E-deficient and vitamin E-sufficient animals in Figure 1. The effect of the vitamin E-deficient diet is clearly evident in the larger percentages of deuterated tocopherol in the rats maintained on that diet. However, the data in Table 1 show that the vitamin E status of the animals does not affect the OH/Ac ratios.

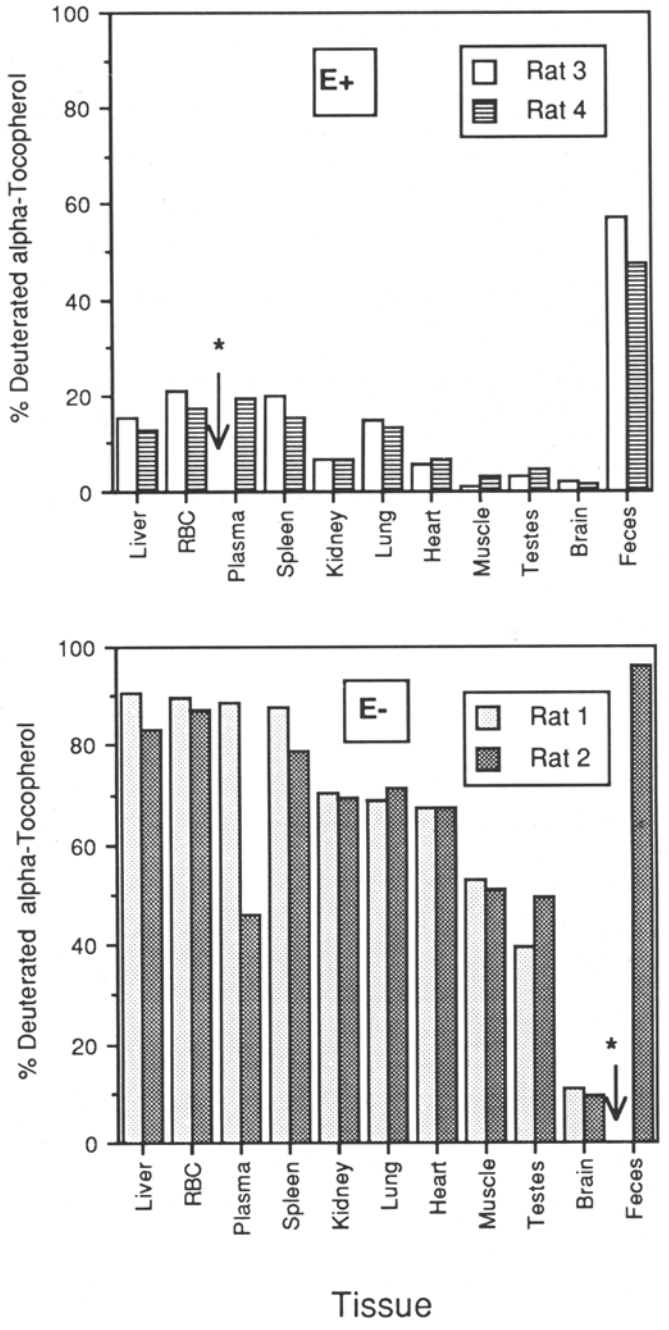

FIG. 1. Percent deuterated α-tocopherol, 100 × [d_3-α-T-OH + d_6-α-T-OH]/[d_0-α-T-OH + d_3-α-T-OH + d_6-α-T-OH], in plasma, RBC, tissues and feces of rats, maintained on vitamin E-deficient (E−) and sufficient (E+) diets, after receiving four daily doses of a mixture of d_6-*RRR*-α-T-OH and d_3-*RRR*-α-T-Ac in tocopherol-stripped corn oil (Experiment 1). An asterisk above or beside a bar denotes a missing value for the tissue indicated.

The data in Table 2 show that free α-tocopherol competed much more effectively with the acetate, when the two compounds were incorporated into an aqueous bolus of laboratory diet (Experiment 2; vitamin E-sufficient rats), than when the two compounds were given in the corn oil. In Experiment 2, the combined mean OH/Ac ratio from the liver, spleen, lung, heart, plasma and RBC at 24 hr was found to be 1.06 ± 0.11 (SEM). Comparison of the OH/Ac values for plasma and RBC at four hr and 24 hr shows that in all but one case the ratio is less at 24 hr than at four hr. This indicates that the proportion of absorbed α-tocopherol derived from the free form declines more rapidly than that derived from the acetate. In the small intestine, the ratios of the original free α-tocopherol (d_6-α-T-OH) to the α-tocopherol (d_3-α-T-OH) derived from the acetate are similar to the OH/Ac ratios in the other tissues, although there is perhaps a slight tendency for this ratio to decrease, from the first segment, closest to the stomach, through to the third, most distant segment. This trend suggests that the hydrolysis of the acetate continues to occur into at least the middle segment of the intestine.

The data for the percent total deuterated α-tocopherol in the plasma and RBC show that although there is little change in the plasma values between four hr and 24 hr, there is a sharp rise in the RBC values over the same period, indicating a time lag in the uptake of tocopherol into RBC. The lag in the uptake into RBC is consistent with the results obtained from two independently conducted in vitro experiments in which it was found that the half-life for the equilibration of α-T-OH between rat plasma and RBC is 2.2 hr at 37 C (16,17).

The mean absolute concentrations of d_0-α-T-OH, d_3-*RRR*-α-T-OH and d_6-*RRR*-α-T-OH measured in the tissues, plasma, RBC, small intestinal segments and feces at 24 hr are presented in Figure 2.

The data obtained from the human study (Experiment 3, Parts A and B) are given in Tables 3 and 4. In the first application of this technology to the measurement of vitamin E uptake in humans (Part A), it is clear from the data in Table 3 that a single dose of 100 mg of deuterated vitamin E is easily detectable in plasma and RBC. The two subjects show that, despite striking differences in the percentages of deuterated α-tocopherol in their plasma and RBCs, the corresponding OH/Ac ratios are remarkably constant over time and between subjects (mean = 1.12 ± 0.04 [SD]), with slightly more absorption of vitamin E occurring from the nonesterified α-tocopherol.

Part B of Experiment 3 illustrates the fact that only one capsule of 100 mg of the mixture of α-tocopherols was required to obtain good data. The results, shown in Table 4, indicate no significant advantage to either form of vitamin E (mean OH/Ac = 0.97 ± 0.04 [SD]). This result agrees well with that obtained in Part A of the experiment. The absolute concentrations of the deuterated and nondeuterated α-tocopherols also are presented in Table 4.

The human data differ markedly from the data obtained with rats dosed according to the traditional bioassay method (Experiment 1), but do agree rather well with the data obtained when the rats were given vitamin E premixed with food (Experiment 2).

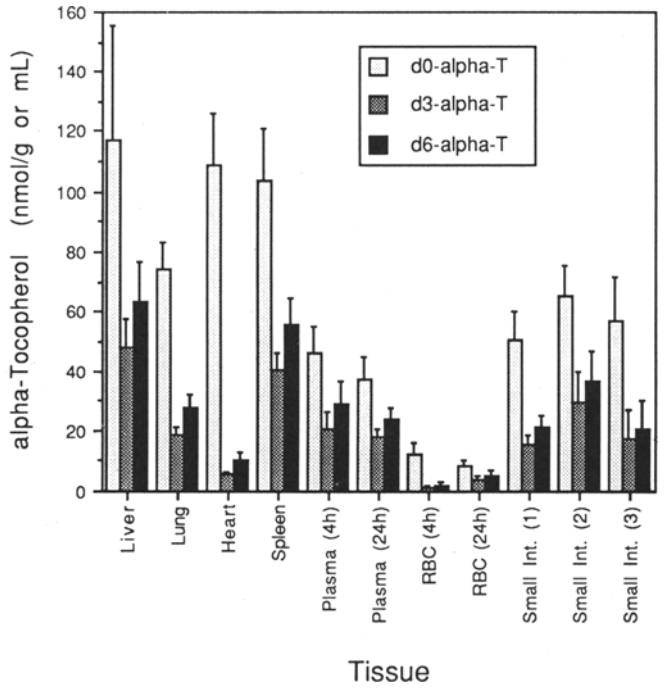

FIG. 2. Mean absolute concentrations of d_0-α-T-OH, d_3-*RRR*-α-T-OH and d_6-*RRR*-α-T-OH in plasma, RBC, tissues and small intestine and contents (divided into thirds) from five rats, four hr (plasma and RBC only) and 24 hr after a single oral dose of d_6-*RRR*-α-T-OH and d_3-*RRR*-α-T-Ac mixed into an aqueous bolus of rat diet (Experiment 2; note: initial d_6/d_3 mole rato = 1.372). Error bars represent one standard deviation. Not shown are the corresponding, substantially larger values for feces which were 176 ± 26, 267 ± 130 and 376 ± 198 nmol/g for the d_0-, d_3- and d_6-α-tocopherols, respectively.

TABLE 3

OH/Ac Ratios (i.e., d_3-α-T-OH/d_6-α-T-OH) and Percent Deuterated α-Tocopherol in Plasma and Red Blood Cells of Two Male, Adult Humans[a]

			OH/Ac[b]		% Deuterated α-tocopherol[c]	
Capsule	Sample	Time	KI	GB	KI	BG
1		0 hr				
	Plasma	15 hr	1.17	1.07	16.6	29.2
	RBC	15 hr	1.09	1.19	9.6	18.3
2		24 hr				
	Plasma	39 hr	1.14	1.07	31.1	39.8
	RBC	39 hr	1.16	1.10	20.4	38.7
	Plasma	87 hr	1.11		25.2	
	RBC	87 hr	1.14		26.7	

[a]During and after oral ingestion, over a two-day period, of two 100-mg doses of an equimolar mixture of d_3-*RRR*-α-T-OH and d_6-*RRR*-α-T-Ac.

[b]Mean OH/Ac (± SD) = 1.12 ± 0.04.

[c]100 × (d_3-α-T-OH + d_6-α-T-OH)/(d_0-α-T-OH + d_3-α-T-OH + d_6-α-T-OH).

TABLE 4

OH/Ac Ratios, Percent Deuterated α-Tocopherol and Absolute Concentrations of α-Tocopherol in Plasma and Red Blood Cells of Three Adult Humans[a]

					Absolute concentrations[d]			
Subject		Elapsed time (hr)	OH/Ac[b]	% Deuterated α-tocopherol[c]	d_0-α-T	d_3-α-T	d_6-α-T	Total
HB	Plasma	18.0	0.98	42.9	26.0	11.0	8.5	45.5
		41.6	0.94	33.7	21.7	6.1	4.9	32.7
	RBC	18.0	0.94	38.9	3.3	1.2	0.9	5.4
		41.6	0.92	36.6	1.7	0.5	0.4	2.7
SL	Plasma	20.0	0.99	41.0	18.5	7.2	5.6	31.3
		43.6	0.99	29.0	17.0	3.9	3.0	23.9
	RBC	20.0	0.93	42.8	3.4	1.4	1.1	5.9
		43.6	0.94	32.4	3.3	0.9	0.7	4.9
DF	Plasma	20.5	1.04	33.1	26.4	7.5	5.5	39.4
		44.1	0.99	27.6	22.8	4.9	3.8	31.5
	RBC	20.5	0.99	27.9	4.7	1.0	0.8	6.6
		44.1	0.99	29.0	3.1	0.7	0.5	4.3

[a]Two females (HB, SL) and one male (DF) following oral ingestion of a single capsule containing 100 mg of a neat mixture of d_3-*RRR*-α-T-OH and d_6-*RRR*-α-T-Ac.

[b]d_3-α-T-OH/d_6-α-T-OH; corrected for initial mole ratio in capsule of 1.310; mean ± SD = 0.97 ± 0.04.

[c]100 × (d_3-α-T-OH + d_6-α-T-OH)/(d_0-α-T-OH + d_3-α-T-OH + d_6-α-T-OH).

[d]nmol/ml of plasma or packed red cells.

DISCUSSION

The advantage of our experimental approach is that the two forms of vitamin E are compared directly within the same subject (rat and human). This eliminates many of the problems associated with interindividual variations, as is clearly and abundantly evident by the consistency of the data we have obtained.

The results from Experiment 1 confirm that the lower activity of nonesterified α-tocopherol observed in bioassays (5–7) is due to the fact that less of it is absorbed from the gut. This result is not affected by the vitamin E status of the animal. However, the much improved relative absorption of the free phenol in Experiment 2 reveals that there is a striking dependence of absorption on the choice of vehicle for delivery of the vitamin.

It has been pointed out that rat diets contain mineral supplements that can promote the oxidation of free tocopherol and that the low potency of the tocopherol, relative to the acetate in rat bioassays, may reflect partial oxidative destruction of the free tocopherol (11,18). Our results, obtained when the vitamin E was mixed in directly with the food, appear, at first, to argue against this suggestion. Thus, even though in Experiment 1 the tocopherol mixture would inevitably come into contact with the rat diet, because dosing occurred over a four-day period, it would be expected that the greatest loss of free tocopherol would occur in Experiment 2, where the free α-tocopherol is exposed to the diet continuously, right from the moment of dosing. (It should be noted that, in order to prevent oxidative losses before dosing, the mixing of the vitamin E with the base diet was delayed until immediately prior to dosing the animals.)

There is, however, the possibility that intestinal oxidative destruction of tocopherol did occur in the animals in Experiment 2, but that it was masked by the operation of other processes. Thus, if hydrolysis of the acetate were rapid compared to the rate of oxidative destruction of the free tocopherol, the effects of oxidation would not be obvious because both forms would be lost at similar rates. Indeed, a rapid hydrolysis of the acetate does appear to have occurred in Experiment 2. Table 2 and Figure 2 show that even as early as four hr after the dose, the amounts of d_3-*RRR*-α-T-OH and d_6-*RRR*-α-T-OH in the plasma are similar (taking into account the differences in the initial proportions of the 2 tocopherols) and are as large as the corresponding values at 24 hr. (However, the d_6-*RRR*-α-T-OH/d_3-*RRR*-α-T-OH ratio is slightly larger at four hr than at 24 hr, indicating that there is some lag in the hydrolysis of the acetate.) Further evidence for a rapid hydrolysis is provided by the d_6-*RRR*-α-T-OH/d_3-*RRR*-α-T-OH ratio obtained for the first third of the small intestine and included contents. The value is essentially the same as that found for the plasma, RBCs and tissues. Furthermore, there is little change, if any, in this ratio in the remaining two-thirds of the small intestine. It appears, therefore, that most of the hydrolysis of the acetate occurred in the first third of the intestine under the conditions of Experiment 2. Also, Figure 2 shows that the absolute amounts of d_3-*RRR*-α-T-OH and d_6-*RRR*-α-T-OH did not vary much between the three small intestinal segments. Another measure of the completeness of hydrolysis was obtained by comparing the d_6-*RRR*-α-T-OH/d_3-*RRR*-α-T-OH ratios in the three, small intestinal segments and in the combined feces (recovered from the large intestine) before and after alkaline hydrolysis. The data, presented in Table 5, show that there is little of the d_3-*RRR*-α-T-OH, if any, remaining after 24 hr.

TABLE 5

Comparison of d_6-α-T-OH/d_3-α-T-OH Ratios, Before and After Alkaline Hydrolysis[a]

	Rat	Before hydrolysis[b]	After hydrolysis[c]	Before/After[d]
Small intestine Segment 1[e]	1	0.96	1.00	0.96
	2	1.05	1.02	1.04
	3	1.03	1.16	0.89
Small intestine Segment 2[e]	1	0.91	0.98	0.93
	2	0.99	0.97	1.02
	3	0.98	1.06	0.92
Small intestine Segment 3[e]	1	0.77	0.78	0.99
	2	1.00	1.04	0.95
	3	1.03	0.94	1.10
Feces	1	0.94	0.76	1.24
	2	1.05	1.01	1.04
	3	1.02	1.13	0.90
	4	1.09	1.02	1.07
	5	1.01	1.00	1.01

[a]In small intestinal segments (three animals) and total fecal material from large intestine (five animals) of rats 24 hr after feeding with a mixture of d_6-*RRR*-α-T-OH and d_3-*RRR*-α-T-Ac in a bolus of food (Experiment 2). Ratios corrected for an initial d_6-*RRR*-α-T-OH/d_3-*RRR*-α-T-Ac mole ratio of 1.372.

[b]These correspond to the OH/Ac ratios in Table 2.

[c]The values will be less than the values before alkaline hydrolysis, if there is d_3-*RRR*-α-T-Ac present. Alkaline hydrolysis was carried out on three equally divided intestinal segments from three of the five rats.

[d]Ratio of the value before hydrolysis to that after hydrolysis. Values which are greater than one indicate the presence of unhydrolyzed d_3-*RRR*-α-T-Ac in the gut 24 hr after receiving the dose.

[e]The small intestine, including contents, was divided into three segments of equal size, beginning with the segment closest to the stomach.

Although a comparable analysis of the small intestine was not conducted in Experiment 1, small but significant amounts of acetate were indeed isolated from fecal material recovered from the large intestine 24 hr after the last dose (Table 1, footnote b). Thus, it is reasonable to infer that hydrolysis of the acetate occurs more slowly when the dose of vitamin E is given in tocopherol-stripped corn oil, leading to a proportionately greater loss of the α-tocopherol originally present as the free phenol.

Oxidative loss, then, still remains the most likely explanation for the poor uptake of free α-tocopherol in Experiment 1. Unfortunately, direct evidence for this phenomenon has, so far, eluded us. Preliminary analyses of fecal material recovered from the large intestine of the rats in Experiment 1 failed to reveal significant amounts of α-tocopheryl quinone, which is, admittedly, only one of several possible oxidation products. Also, this quinone was not detected in the plasma of these animals.

The small variations in the OH/Ac values observed between tissues from the same animal in Experiment 1 may be real, because the proportions of α-tocopherol from the free phenol source and the acetate source passing across

the intestinal wall into the lymph may have varied with time, due to the lag in the hydrolysis of the acetate. Furthermore, it is likely that different tissues absorbed α-tocopherol from plasma at differing rates during the course of the experiment (12).

The competitive uptake of α-T-OH into human blood from a mixture of free α-tocopherol and α-tocopheryl acetate consumed during a regular evening meal shows that the free form, on a mole-for-mole basis, is at least equal to the acetate in bioavailability. This finding, which is in agreement with the earlier studies (9–11), is the most direct and strongest support for maintaining the existing USP standard for the relative biopotencies of α-tocopherol and α-tocopheryl acetate. That is, these two forms should continue to be accorded equal potency on a molar basis.

Although data were obtained from only five human subjects, the results for the relative uptake of the two forms of vitamin E show remarkably little variation, suggesting that dietary status, type of meal, sex and other factors had relatively little influence on the result.

Finally, these experiments illustrate the need for caution in relying on results obtained from laboratory animals as a guide to relative potencies of different forms of tocopherol in human nutrition.

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REFERENCES

1. Diplock, A.T. (1985) in *Fat Soluble Vitamins: Their Biochemistry and Applications* (Diplock, A.T., ed.) pp. 154–224, Heinemann, London, England.
2. Gallo-Torres, H.E. (1980) in *Vitamin E* (Machlin, L.J., ed.) pp. 170–192, Marcel Dekker, New York.
3. Nierenberg, D.W., Lester, D.C., and Colacchio, T.A. (1987) *J. Chromatogr. 413,* 79–89.
4. Nierenberg, D.W., and Lester, D.C. (1985) *J. Chromatogr. 345,* 275–284.
5. Harris, P.L., and Ludwig, M.I. (1949) *J. Biol. Chem. 180,* 611–614.
6. Leth, T., and Sondergaard, H. (1983) *Internat. J. Vit. Nutr. Res. 53,* 297–311.
7. Wieser, H., Vecchi, M., and Schlachter, M. (1986) *Int. J. Vit. Nutr. Res. 56,* 45–56.
8. USP XXI: The Pharmacopeia of the United States of America. NF XVI: The National Formulary, pp. 1118–1121. United States Pharmacopeial Convention, Inc., Rockville, MD 1984.
9. Week, E.F., Sevigne, F.J., and Ellis, M.E. (1952) *J. Nutr. 46,* 353–359.
10. Baker, H., Frank, O., DeAngelis, B., and Feingold, S. (1980) *Nutr. Rep. Int. 21,* 531–536.
11. Horwitt, M.K., Elliott, W.H., Kanjananggulpan, P., and Fitch, C.D. (1984) *Am. J. Clin. Nutr. 40,* 240–245.
12. Ingold, K.U., Burton, G.W., Foster, D.O., Hughes, L., Lindsay, D.A., and Webb, A. (1987) *Lipids 22,* 163–172.
13. Ingold, K.U., Hughes, L., Slaby, M., and Burton, G.W. (1987) *J. Labelled Comp. Radiopharm. 24,* 817–831.
14. Burton, G.W., Webb, A., and Ingold, K.U. (1985) *Lipids 20,* 29–39.
15. Bieri, J.G., Stoewsand, G.S., Briggs, G.M., Phillips, R.W., Woodard, J.C., and Knapka, J.J. (1977) *J. Nutr. 107,* 1340–1348.
16. Bjornson, L.K., Gniewkowski, C., and Kayden, H.J. (1975) *J. Lipid. Res. 16,* 39–53.
17. Cheng, S.C., Burton, G.W., Ingold, K.U., and Foster, D.O. (1987) *Lipids 22,* 469–473.
18. Horwitt, H.K. (1986) Editorial note in *Vitamin E: 1986 Abstracts,* pp. 68–69, VERIS, La Grange, IL.

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Influence of Dietary Fats on Pancreatic Phospholipids of Chronically Ethanol-treated Rats

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Male rats were given liquid diets by pair-feeding for 24–30 days, and phosphatidylinositols in pancreas were analyzed as derivatives of diacylglycerols and fatty acids. Addition of arachidonic acid or changing the fat component (35 energy%) in the liquid diet from olive oil/corn oil to oil from *Borago officinalis*, which contains 22% γ-linolenic acid, increased the fraction of arachidonoyl-containing species. This fraction was decreased by more than 50% by substituting ethanol for 36 of the 47 energy% provided by carbohydrate. A smaller difference between ethanol-fed and control rats was seen in the composition of phosphatidylcholines and phosphatidylethanolamines. There was no difference in the composition of phosphatidylinositols when fat, instead of ethanol, was used to substitute the 36 energy% in the diet containing olive oil/corn oil. Substituting ethanol for 28 of 35 energy% provided by fat as corn oil in a liquid diet had no effect on the fraction of arachidonoyl-containing species. The results indicate that the effect of ethanol on phosphatidylinositols in pancreas is not due to a deficiency of arachidonic acid, and that the effect of the ethanol-containing diet is not due to the lowered carbohydrate content. However, high contents of fat or of ethanol appear to be necessary for the effect.
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Chronic ethanol abuse may cause pancreatitis in man. The pathogenesis is incompletely understood, and this hampers rational treatment and prevention. Studies on chronic effects of ethanol are commonly performed by feeding rats an ethanol-containing liquid diet (1). Using this model, it was found that the arachidonoyl-containing phosphatidylinositols in the pancreas were replaced with other molecular species to a large extent (2,3). Phosphatidylinositols are involved in the mediation of secretion responses upon stimulation (4,5). It was therefore considered that the change in composition could be related to the pathogenesis of secretion disturbances and pancreatitis.

The decrease of arachidonoyl-containing phosphatidylinositols might have been due to deficiency of arachidonic acid, e.g., due to increased production of eicosanoids (6,7). Diets rich in γ-linolenic acid (all-*cis*-6,9,12-octadecatrienoic acid), a precursor of arachidonic acid, might protect against some detrimental effects of ethanol (7–9). Administration of arachidonic acid also has been shown to protect against the ethanol-induced fatty liver (7,8,10,11). Liquid ethanol-containing diets therefore were enriched with these fatty acids in order to study if this protects against the biochemical changes in the pancreas.

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Abbreviations: γ-linolenic acid, all-*cis*-6,9,12-octadecatrienoic acid; GLC, gas liquid chromatography.

The liquid-diet model described by DeCarli and Lieber (12) involves administration of much more carbohydrate to the control rats than to the ethanol-fed rats and this might, in fact, be the reason for several of the differences seen between the ethanol-fed and the control rats (13). A control experiment, therefore, was designed to study the effect of isocaloric substitution of the carbohydrate in the control diet with fat. A high-fat content in the diet might be necessary for the chronic effects of ethanol on the digestive enzymes in the pancreas (14,15). The effect of ethanol on the phospholipid composition, therefore, also was studied in an experiment using a low-fat content in the ethanol-containing diet.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats were used. The body weights are given in Tables 2 and 6–8. Alcohol and control liquid diets according to DeCarli and Lieber (12) and the same diets without the fat component were obtained from Bioserv, Inc. (Frenchtown, NJ). Oil obtained by hexane-extraction of seeds from *Borago officinalis* was prepared by BioOil Research Ltd. (Crewe, England), analyzed by Dr. Bengt Herslöw, KabiVitrum, Stockholm, Sweden, and found to contain 11.8% palmitic, 3.7% stearic, 16.9% oleic, 38.0% linoleic, 22.1% γ-linolenic, 3.6% eicosanoic, 1.0% docosahexanoic and 2.0% erucic acid by weight. Arachidonic acid (>90% pure) was obtained from NuChek Prep, Inc. (Elysian, MN).

Diets. The composition of the diets is shown in Table 1. The diets used in Experiments 1–3 were based on the alcohol and control diets described by DeCarli and Lieber (12). The diets used in Experiment 4 were based on the low-fat diets described by Thompson and Reitz (16). The energy content was 1.24 kcal/ml, as compared to 1 kcal/ml in the DeCarli-Lieber diet, and the major nutrients were lactalbumin (Sigma, St. Louis, MO), sucrose and corn oil. These diets also contained citric acid, vitamins and salts (16) and Tween-80 (Sigma), 0.1 g/g fat.

Animal experiments. The rats were pair-fed, i.e., the rats eating the smallest volume on one day determined the provision of diet to the other rats in each pair or group on the following day. The diets were given for 24 days (Expts. 1–3) or 30 days (Expt. 4). The mean intake of ethanol in the different groups was 12.3–13.7 $g \times kg^{-1} \times day^{-1}$. To ensure that control rats and rats fed ethanol were equally nourished at the time of sacrifice (09.00), the diets were changed both at 09.00 and 24.00 on the day before sacrifice. The rats were killed by cervical dislocation, and the pancreas and liver were taken out, avoiding visible fat.

Analytical procedures. The contents of triacylglycerols and phospholipids in the liver were determined by extraction and chromatography as previously described (11). The species compositions of glycerophosphatides in pancreas were quantitatively determined by gas liquid chromatography (GLC) of 1,2-diacylglycerol 3-trimethylsilyl

TABLE 1

Composition of Diets

Expt. no.	Major nutrients				Source of fat
	Ethanol	Protein	Carbohydrate	Fat	
	energy%				
1	0/36	18	47/11	35	O+C[a]
1	0/36	18	47/11	35	*Borago* oil
2[b]	0/36	18	47/11	35	97.5% O+C[a] + 2.5% 18:1
2[b]	0/36	18	47/11	35	97.5% O+C[a] + 2.5% 20:4
3	0	18	47/11	35/71	O+C[a]
4	0/28	18	47	35/7	O+C[a]

[a]Olive oil (28.4 g/l), corn oil (8.5 g/l) and ethyl linoleate (2.7 g/l),
[b]diets containing di-tert-butyl-p-cresol (BHT), 4 mg/l.

ethers (2,3,17). The major acyl groups in pancreatic phospholipids were determined by GLC of methyl esters obtained by transmethylation in boron trifluoride-methanol (18), using a fused silica capillary column coated with methyl silicone. Student's t-test was used for statistical analysis.

RESULTS

Effects of diets rich in γ-linolenic acid (Expt. 1). The content of hepatic triacylglycerol was markedly and significantly higher in the rats fed the ethanol-containing diets than in the control diets (Table 2). This was true both when the diet contained the olive oil/corn oil mixture (12) and when it contained the *Borago* oil, rich in γ-linolenic acid. The content of hepatic phospholipids was the same in the different groups.

The fatty acyl composition of the pancreatic phospholipids was significantly different in rats fed the *Borago* oil and in rats fed olive oil/corn oil (Tables 3-5). In all three phospholipid classes, the fraction of arachidonoyl groups was higher and the fraction of oleoyl groups lower in the group fed *Borago* oil. The fraction of stearoyl groups in phosphatidylethanolamine and phosphatidylcholine and the fraction of palmitoyl groups in phosphatidylcholine were higher in the rats fed *Borago* oil. The results were in agreement with those obtained by analysis of molecular species. Thus, both the 36:4 and 38 species of phosphatidylcholines and phosphatidylinositols, consisting mainly of the 1-palmitoyl- and 1-stearoyl-2-arachidonoyl species, respectively (2), constituted larger fractions in the rats fed *Borago* oil. The 34:1-2 fractions contain mainly the 1-palmitoyl-2-oleoyl species and the 36:1-2 fraction the 1-stearoyl-2-oleoyl and 1-stearoyl-2-linoleoyl species (3). These fractions were lower in the rats fed the *Borago* oil.

The composition of phosphatidylinositols was markedly and significantly different in the pancreas of rats fed the control diet and the ethanol-containing diet which both contained olive oil/corn oil (12) (Table 3). The fraction of the 36:4 species, consisting mainly of the palmitoyl-arachidonoyl species, was about 80% lower, and the fraction of the 38:3-4 species (mainly the 1-stearoyl-2-arachidonoyl), 50% lower in the ethanol-fed rats. These results are confirmed by the decreased content of arachidonoyl groups in the ethanol-fed rats. Corresponding differences in the opposite direction were seen for the relative content of oleoyl groups and for the 34:0 species (probably palmitoyl-stearoyl) and the 36:1-2 species. Similar differences between ethanol-fed and control rats were seen with the rats fed the *Borago* oil. However, the relative content of oleoyl groups was less markedly elevated in the ethanol-fed rats among the rats given *Borago* oil. Instead, the relative content of linoleoyl groups was markedly elevated. In accordance with this, the 36:2-3 species, which contain linoleoyl groups, constituted a larger fraction in these ethanol-fed rats than in the corresponding controls.

The composition of phosphatidylcholines was significantly different in pancreas from rats fed the control diet and the ethanol diet containing olive oil/corn oil (12) (Table 4). Thus, the 36:1-2 fraction and the content of stearoyl and linoleoyl groups were higher in the ethanol-fed rats. There were no marked differences in the content of arachidonoyl-containing species. However, such differences were seen between the control rats and ethanol-fed rats that had been given the diets containing *Borago* oil. Thus, the 36:4 fraction was much lower in the ethanol-fed rats, which caused the difference between the groups given *Borago* oil and olive oil/corn oil to disappear. The difference also was seen from the acyl group composition. The content of linoleoyl groups was higher, and that of arachidonoyl groups was lower, in the ethanol-fed rats.

The acyl group composition in the phosphatidylethanolamine fraction was significantly different in pancreas from control rats and ethanol-fed rats (Table 5). The differences were essentially the same in the rats fed the diets containing olive oil/corn oil (12) and in the rats fed the diets containing *Borago* oil. The relative content of the saturated acyl groups was lower, and that of the oleoyl and linoleoyl groups higher, in the rats given ethanol. The relative content of arachidonoyl groups was the same in control rats and in ethanol-fed rats.

Effects of diets containing arachidonic acid (Expt. 2). The species composition of phosphatidylinositols in pancreas was analyzed in rats fed the liquid ethanol-containing and control diets (12) supplemented with arachidonic acid or oleic acid (Table 6). The 38:3-4 fraction was

TABLE 2

Hepatic Lipids of Rats Fed Liquid Diets With and Without Ethanol and With Different Fat Components (Expt. 1)

Hepatic lipids (mg/g)	Olive oil/corn oil diet		*Borago* oil (22.1% GLA) diet	
	Control	Ethanol	Control	Ethanol
Triacylglycerols	9.6 ± 1.7	82.4 ± 19.3[c]	15.7 ± 5.8[a]	76.3 ± 30.1[c]
Phospholipids	28.0 ± 0.9	30.5 ± 7.1	27.6 ± 2.6	24.4 ± 3.0
Body wt (g)				
Day 1	142.0 ± 3.6	142.2 ± 2.8	142.8 ± 3.5	143.4 ± 2.7
Day 24	232.1 ± 5.8	201.3 ± 12.6[b]	232.7 ± 4.2	187.7 ± 19.3[c]

Results are expressed as mean ± S.D. of values from six rats.

[a]$p < 0.05$,

[b]$p < 0.01$,

[c]$p < 0.001$ in comparisons of control and ethanol-fed rats and in comparisons of rats fed the two control diets.

TABLE 3

Percentage Composition of Phosphatidylinositols in Pancreas of Rats Fed Liquid Diets With and Without Ethanol and With Different Fat Components (Expt. 1)

Acyl carbons: double bonds	Olive oil/corn oil diet		*Borago* oil (22.1% GLA) diet	
	Control	Ethanol	Control	Ethanol
Species composition				
32:1	0.4 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.2
32:0	2.4 ± 0.2	2.4 ± 0.5	2.6 ± 0.6	2.8 ± 0.4
34:1-2	9.5 ± 0.6	9.9 ± 1.7	7.3 ± 1.1[b]	8.7 ± 1.4
34:0	5.2 ± 0.4	12.2 ± 1.6[c]	4.6 ± 0.4[a]	12.1 ± 0.8[c]
36:4	5.6 ± 0.4	1.1 ± 0.6[c]	7.8 ± 0.5[c]	1.6 ± 0.1[c]
36:2-3	6.1 ± 0.6	6.9 ± 1.2	6.4 ± 0.4	9.3 ± 1.7[b]
36:1-2	22.4 ± 1.8	44.9 ± 3.5[c]	16.9 ± 1.2[c]	39.9 ± 1.4[c]
38:3-4	47.2 ± 2.3	20.3 ± 4.5[c]	53.0 ± 3.0[b]	23.4 ± 2.2[c]
40:[d]	1.2 ± 0.8	1.9 ± 1.1	1.2 ± 0.3	1.9 ± 1.3
Acyl group composition				
16:0	20.6 ± 3.4	21.1 ± 4.1	21.9 ± 3.6	21.0 ± 2.4
18:2	7.6 ± 1.8	9.2 ± 1.7	6.6 ± 0.7	14.4 ± 2.0[c]
18:1	16.3 ± 1.0	25.6 ± 2.3[c]	10.6 ± 1.3[c]	15.3 ± 2.2[b]
18:0	37.3 ± 2.0	37.8 ± 2.6	39.0 ± 2.5	41.3 ± 2.7
20:4	18.3 ± 2.0	6.2 ± 1.4[c]	22.0 ± 2.0[b]	8.0 ± 1.2[c]

Results are expressed as mean ± S.D. of values from six rats.

[a]$p < 0.05$,

[b]$p < 0.01$,

[c]$p < 0.001$ in comparisons of control and ethanol-fed rats and in comparisons of rats fed the two control diets.

[d]Number of double bonds not determined.

increased in the control rats given the arachidonic acid-supplemented diet. The differences between the compositions seen in control rats and ethanol-fed rats were about the same when the diets were unsupplemented (Table 3) or supplemented with oleic acid or arachidonic acid. The relative content of arachidonoyl-containing species in ethanol-treated rats was higher in experiments with arachidonoyl-containing diets than in experiments with diets supplemented with oleic acid.

Comparison of energy substitution with lipid and with carbohydrate (Expt. 3). Rats were pair-fed the control diet described by DeCarli and Lieber (12), which contains carbohydrate instead of ethanol, and a corresponding control diet with fat instead of ethanol. The species composition of the phosphatidylinositols in pancreas was about the same in these two groups of controls (Table 7).

Effects of ethanol addition to a low-fat diet (Expt. 4). Phosphatidylinositols in pancreas were analyzed both

TABLE 4

Percentage Composition of Phosphatidylcholines in Pancreas of Rats Fed Liquid Diets With and Without Ethanol and With Different Fat Components (Expt. 1)

Acyl carbons: double bonds	Olive oil/corn oil diet		*Borago* oil (22.1% GLA) diet	
	Control	Ethanol	Control	Ethanol
Species composition				
32:1	1.0 ± 0.1	0.6 ± 0.1[c]	0.8 ± 0.1	0.7 ± 0.4
32:0	3.9 ± 0.2	2.5 ± 0.3[c]	5.5 ± 0.3[c]	4.0 ± 0.6[c]
34:2-3	1.0 ± 0.2	0.7 ± 0.1[a]	3.1 ± 0.3[c]	2.9 ± 0.5
34:1-2	42.1 ± 1.5	43.4 ± 5.3	31.9 ± 1.0[c]	36.9 ± 1.7[c]
36:4	14.2 ± 0.4	11.9 ± 2.0[a]	20.6 ± 0.7[c]	14.6 ± 1.1[c]
36:[d]	6.3 ± 1.1	4.7 ± 1.0[a]	7.2 ± 0.9	5.3 ± 0.9[b]
36:2-3	14.2 ± 0.6	13.5 ± 0.8	12.3 ± 0.5[c]	13.6 ± 0.9[a]
36:1-2	8.6 ± 0.3	12.4 ± 1.0[c]	8.0 ± 0.3[b]	11.0 ± 1.2[c]
38:[d]	8.7 ± 1.1	10.3 ± 3.1	10.5 ± 0.9[a]	10.8 ± 1.2
40:[d]	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.2	0.2 ± 0.2
Acyl group composition				
16:0	40.9 ± 2.2	37.5 ± 1.1	44.5 ± 1.4[b]	40.9 ± 1.7[b]
18:2	20.1 ± 1.1	24.8 ± 3.4[b]	16.0 ± 0.9[c]	23.7 ± 1.3[c]
18:1	15.9 ± 1.1	15.0 ± 0.7	8.8 ± 1.1[c]	8.1 ± 1.0
18:0	6.4 ± 0.5	8.2 ± 1.2[b]	7.5 ± 0.5[b]	8.9 ± 1.1[a]
20:4	16.6 ± 1.6	14.5 ± 3.6	23.2 ± 1.6[c]	18.5 ± 1.3[c]

Results are expressed as mean ± S.D. of values from six rats.

[a]$p < 0.05$,

[b]$p < 0.01$,

[c]$p < 0.001$ in comparisons of control and ethanol-fed rats and in comparisons of rats fed the two control diets.

[d]Number of double bonds not determined.

TABLE 5

Percentage Acyl Group Composition of Phosphatidylethanolamines in Pancreas of Rats Fed Liquid Diets With and Without Ethanol and With Different Fat Components (Expt. 1)

Acyl carbons: double bonds	Olive oil/corn oil diet		*Borago* oil (22.1% GLA) diet	
	Control	Ethanol	Control	Ethanol
16:0	14.4 ± 1.0	9.0 ± 1.3[c]	14.7 ± 1.4	9.5 ± 1.2[c]
18:2	9.7 ± 0.4	12.5 ± 1.8[b]	8.6 ± 1.1[a]	13.3 ± 0.6[c]
18:1	21.1 ± 0.9	28.6 ± 2.1[c]	13.7 ± 1.1[c]	20.1 ± 3.6[b]
18:0	20.1 ± 1.2	18.7 ± 1.1	23.7 ± 2.0[b]	20.0 ± 1.2[b]
20:4	34.7 ± 2.4	31.2 ± 3.4	39.3 ± 4.4[a]	37.2 ± 3.0

Results are expressed as mean ± S.D. of values from six rats.

[a]$p < 0.05$,

[b]$p < 0.01$,

[c]$p < 0.001$ in comparisons of control and ethanol-fed rats and in comparisons of rats fed the two control diets.

with respect to species composition and acyl group composition (Table 8). No differences were observed between the two groups in the content of the arachidonoyl group or the arachidonoyl-containing species. The content of linoleoyl groups and linoleoyl-containing species was lower in the ethanol-fed rats, and the linoleoyl group was then replaced by oleoyl and palmitoyl groups.

DISCUSSION

Very pronounced decreases have been observed previously for arachidonoyl-containing phosphatidylinositols in pancreas of rats fed an ethanol-containing liquid diet (2,3). These results were confirmed in the present study (Table 3). This effect of ethanol might have been due to

TABLE 6

Percentage Composition of Phosphatidylinositol Species in Pancreas of Rats Fed Liquid Diets With and Without Ethanol and Supplemented With Oleic Acid or Arachidonic Acid, 2.5% of the Fat (Expt. 2)

Acyl carbons: double bonds	Oleic acid diets		Arachidonic acid diets	
	Control	Ethanol	Control	Ethanol
32:1	0.4 ± 0.1	0.3 ± 0.1[b]	0.3 ± 0.1	0.3 ± 0.1
32:0	2.4 ± 0.4	1.9 ± 0.3	1.8 ± 0.3[a]	1.6 ± 0.2
34:1-2	12.4 ± 1.2	11.2 ± 0.9	9.4 ± 0.9[b]	10.2 ± 0.8
34:0	5.8 ± 0.9	13.5 ± 1.4[c]	5.2 ± 0.6	11.7 ± 1.4[c]
36:4	5.7 ± 0.7	0.9 ± 0.1[c]	6.3 ± 0.4	1.3 ± 0.3[c]
36:2-3	8.0 ± 0.5	7.6 ± 0.5	6.7 ± 0.9[a]	7.6 ± 1.2
36:1-2	22.4 ± 1.0	46.6 ± 1.3[c]	21.2 ± 1.5	43.9 ± 6.0[c]
38:3-4	42.4 ± 2.8	16.6 ± 1.3[c]	48.8 ± 1.3[b]	21.8 ± 4.4[c]
40:[d]	0.5 ± 0.6	1.6 ± 0.2[a]	0.3 ± 0.3	1.8 ± 0.8[a]
Body wt (g)				
Day 1	134.0 ± 5.5	134.5 ± 4.0	133.8 ± 4.6	134.3 ± 3.2
Day 24	228.0 ± 13.4	209.8 ± 30.1	224.1 ± 6.5	200.3 ± 7.3[c]

Results are expressed as mean ± S.D. of values from four rats.

[a]$p < 0.05$,

[b]$p < 0.01$,

[c]$p < 0.001$ in comparisons of control and ethanol-fed rats and in comparisons of rats fed the two control diets.

[d]Number of double bonds not determined.

TABLE 7

Percentage Composition of Phosphatidylinositol Species in Pancreas of Rats Fed a Liquid Control Diet Used in Ethanol Feeding Experiments* (Expt. 3)

Acyl carbons: double bonds	Carbohydrate substitution	Fat substitution
32:1	0.4 ± 0.1	0.2 ± 0.0[a]
32:0	1.9 ± 0.3	1.9 ± 0.2
34:1-2	10.1 ± 0.8	9.8 ± 0.8
34:0	6.3 ± 0.3	7.0 ± 0.7
36:4	3.8 ± 0.2	2.9 ± 0.6[a]
36:2-3	7.0 ± 1.1	6.5 ± 0.8
36:1-2	26.6 ± 2.7	31.8 ± 5.4
38:3-4	43.9 ± 4.0	39.7 ± 6.4
40:[d]	0.1 ± 0.1	0.1 ± 0.0
Body wt (g)		
Day 1	113.4 ± 3.0	113.6 ± 3.8
Day 24	298.9 ± 22.1	310.6 ± 8.9

*36 Energy% from ethanol was substituted by carbohydrate and the same diet with this substitution done by fat.

Results are expressed as mean ± S.D. of values from five rats.

[a]$p < 0.05$ in comparisons of the two groups.

[b,c]Not applicable.

[d]Number of double bonds not determined.

deficiency of arachidonic acid (6-8). Since prostaglandins might protect against pancreatitis (19), a vicious circle was suggested to explain the effects (17). Thus, the diets were supplemented with arachidonic acid or its precursor γ-linolenic acid. The results indicate that the decrease in arachidonoyl-containing phosphatidylinositols in the pancreas is not due to insufficient supply of arachidonic acid or to reduced activity of the Δ^6-desaturase (7,20).

TABLE 8

Percentage Composition of Phosphatidylinositols in Pancreas of Rats Fed Liquid Diets* (Expt. 4)

Acyl carbons: double bonds	Control	Ethanol
Species composition		
32:1	0.3 ± 0.1	1.1 ± 0.1[c]
32:0	3.6 ± 0.4	5.8 ± 0.9[c]
34:1-2	9.1 ± 1.0	15.6 ± 1.3[c]
34:0	8.1 ± 0.5	10.5 ± 1.9[a]
36:4	3.7 ± 0.5	4.5 ± 0.8
36:2-3	4.1 ± 0.3	3.8 ± 0.5
36:1-2	34.9 ± 1.6	22.1 ± 2.0[c]
38:3-4	35.5 ± 2.0	35.4 ± 4.4
40:[d]	0.7 ± 0.4	1.2 ± 0.4
Acyl group composition		
16:0	16.0 ± 1.2	21.7 ± 1.8[c]
18:2	16.6 ± 0.9	7.5 ± 0.8[c]
18:1	5.5 ± 0.8	9.9 ± 1.0[c]
18:0	41.2 ± 1.0	39.8 ± 1.2[a]
20:4	20.8 ± 1.7	21.1 ± 2.5
Body wt (g)		
Day 1	118.5 ± 3.3	117.2 ± 2.6
Day 30	235.2 ± 9.1	224.8 ± 4.8

*47 Energy% from carbohydrate and either 35 energy% from fat or 7 energy% from fat and 28 from ethanol.

Results are expressed as mean ± S.D. of values from five rats.

[a]$p < 0.05$ in comparisons of the two groups.

[b]Not applicable.

[c]$p < 0.001$ in comparisons of the two groups.

[d]Number of double bonds not determined.

In a previous study, no significant differences were seen in the composition of phosphatidylcholines and phosphatidylethanolamines in the pancreas of rats fed ethanol-containing and control diets (2). A more detailed analysis revealed significant differences in the compositions, but these were smaller than for the phosphatidylinositols (Tables 3–5). The differences seen between ethanol-fed and control rats were more marked when the diet was based on the γ-linolenic acid-containing *Borago* oil than when it was based on olive oil/corn oil (12).

It is difficult to assess the effect of these changes on the physical properties of the membrane. The changes in the composition of phosphatidylcholines and phosphatidylethanolamines do not appear to be consistent with a decrease in fluidity (21,22). However, it was recently reported that phosphatidylinositols specifically confer membrane tolerance to ethanol when obtained from ethanol-fed rats (23). This is consistent with the pronounced changes in the composition of this phospholipid in ethanol-treated rats.

One aim of the present study was to evaluate if the composition of phosphatidylinositols could be changed by dietary means, since this might protect against the effects of ethanol on pancreas. Diets containing oil from evening primrose *(Oenothera biennis)* have been suggested for prevention of various diseases (24), including those caused by ethanol (8,9), and the active component is considered to be γ-linolenic acid. The methyl ester of this attenuates the ethanol-induced fatty liver when administered intraperitoneally (8,25). Since oil from *Borago officinalis* has more than twice the γ-linolenic acid content of evening primrose oil (9), it was chosen for the present study. However, the dietary, rather than the intraperitoneal, administration of larger amounts of γ-linolenic acid did not attenuate the hepatic accumulation of triacylglycerols in the ethanol-fed rats (Table 2). Possibly, this was due to the difference in mode of administration or in the molecular species composition of the oils.

The rats fed the *Borago* oil had elevated fractions of arachidonoyl-containing phospholipids in pancreas. The changes in the phosphatidylinositols were small and it is not known if they might have had any effect on the function, e.g., in the phosphoinositide cycle (5) or the attachment of acetylcholine esterase (26). Diets containing arachidonic acid and *Borago* oil had about the same effects on the composition, in agreement with efficient conversion of γ-linolenic to arachidonic acid.

The differences between the compositions of the phospholipids in ethanol-fed and control rats were not eliminated by the *Borago* oil. However, the relative content of arachidonoyl-containing phosphatidylcholines was the same, in the control rats fed the olive oil/corn oil diet, as in the rats fed the ethanol-containing diet based on *Borago* oil. Thus, this oil might protect against effects of ethanol which are due to certain membrane changes (21) and to general deficiencies of arachidonic acid (7,9,24).

When rats are pair-fed liquid ethanol-containing diets, the energy provided by ethanol (36%) is usually substituted with carbohydrate in the control diets (12). Feeding two control diets that differed with respect to fat and carbohydrate content indicated that the effect of the ethanol-containing diet was not due solely to the low content of carbohydrate. However, when the diets contained equal and high amounts of carbohydrate (47 energy%), there was no difference in the arachidonoyl-containing fraction of phosphatidylinositols between rats given 35 energy% as fat and rats given a diet providing 28 energy% from ethanol and 7 energy% from fat. Thus, high contents of fat or of ethanol appear to be necessary for the effect on the phosphatidylinositol composition. This is similar to the requirements for effects on the secretory function of the pancreas (14,15) and accumulation of fat in the liver (27–29).

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REFERENCES

1. Lieber, C.S., and DeCarli, L.M. (1982) *Alcoholism Clin. Exp. Res. 6,* 523–531.
2. Cronholm, T., and Curstedt, T. (1984) *Biochem. Pharmacol. 33,* 1105–1109.
3. Cronholm, T., and Curstedt, T. (1985) *Alcohol 2,* 183–186.
4. Hokin, L.E., and Hokin, M.R. (1955) *Biochim. Biophys. Acta 18,* 102–110.
5. Berridge, M.J. (1986) *J. Cell Sci. Suppl. 4,* 137–153.
6. Alling, C., Becker, W., Jones, A.W., and Änggård, E. (1984) *Alcoholism Clin. Exp. Res. 8,* 238–242.
7. Horrobin, D.F. (1987) *Alcoholism Clin. Exp. Res. 11,* 2–9.
8. Cunnane, S.C. (1987) *Alcoholism Clin. Exp. Res. 11,* 25–31.
9. Glen, I., Skinner, F., Glen, E., and MacDonell, L. (1987) *Alcoholism Clin. Exp. Res. 11,* 37–41.
10. Goheen, S.C., Larkin, E.C., Manix, M., and Rao, G.A. (1980) *Lipids 15,* 328–336.
11. Karpe, F., Wejde, J., and Änggård, E. (1984) *Acta Pharmacol. Toxicol. 55,* 95–99.
12. DeCarli, L.M., and Lieber, C.S. (1967) *J. Nutr. 91,* 331–336.
13. Gonzalez-Calvin, J.L., Saunders, J.B., Crossley, I.R., Dickenson, C.J., Smith, H.M., Tredger, J.M., and Williams, R. (1985) *Biochem. Pharmacol. 34,* 2685–2689.
14. Ponnappa, B.C., Hoek, J.B., Sarchet, K., and Rubin, E. (1986) *J. Lab. Clin. Med. 107,* 556–562.
15. Singh, M. (1987) *Dig. Dis. Sci. 32,* 57–64.
16. Thompson, J.A., and Reitz, R.C. (1978) *Lipids 13,* 540–550.
17. Cronholm, T., and Curstedt, T. (1986) *Biochem. Pharmacol. 35,* 1608–1611.
18. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600–608.
19. Coelle, E.F., Adham, N., Elashoff, J., Lewin, K., and Taylor, I.L. (1983) *Gastroenterology 85,* 1307–1312.
20. Nervi, A.M., Peluffo, R.O., Brenner, R.R., and Leikin, A.I. (1980) *Lipids 15,* 263–268.
21. Sun, G.Y., and Sun, A.Y. (1985) *Alcoholism Clin. Exp. Res. 9,* 164–180.
22. Stubbs, C.D., and Smith, A.D. (1984) *Biochim. Biophys. Acta 779,* 89–137.
23. Taraschi, T.F., Ellingson, J.S., Wu, A., Zimmerman, R., and Rubin, E. (1976) *Proc. Natl. Acad. Sci. USA 83,* 9398–9402.
24. Booyens, J., and van der Merwe, C.F. (1985) *Medical Hypotheses 18,* 53–60.
25. Segarnick, D.J., Cordasco, D.M., Agura, V., Cooper, N.S., and Rotrosen, J. (1985) *Prostagl. Leukotr. Med. 17,* 277–282.
26. Low, M.G., Ferguson, M.A.J., Futerman, A.H., and Silman, I. (1986) *Trends Biochem. Sci. 11,* 212–215.
27. Lieber, C.S., and DeCarli, L.M. (1970) *Am. J. Clin. Nutr. 23,* 474–478.
28. Rao, G.A., and Larkin, E.C. (1984) *Nutr. Res. 4,* 903–912.
29. Rao, G.A., Riley, D.E., and Larkin, E.C. (1987) *Nutr. Res. 7,* 81–87.

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Differential Effects of Dietary Linoleic and α-Linolenic Acid on Lipid Metabolism in Rat Tissues

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Comparative effects of feeding dietary linoleic (safflower oil) and α-linolenic (linseed oil) acids on the cholesterol content and fatty acid composition of plasma, liver, heart and epididymal fat pads of rats were examined. Animals fed hydrogenated beef tallow were used as isocaloric controls. Plasma cholesterol concentration was lower and the cholesterol level in liver increased in animals fed the safflower oil diet. Feeding the linseed oil diet was more effective in lowering plasma cholesterol content and did not result in cholesterol accumulation in the liver. The cholesterol concentration in heart and the epididymal fat pad was not affected by the type of dietary fatty acid fed. Arachidonic acid content of plasma lipids was significantly elevated in animals fed the safflower oil diet and remained unchanged by feeding the linseed oil diet, when compared with the isocaloric control animals fed hydrogenated beef tallow. Arachidonic acid content of liver and heart lipids was lower in animals fed diets containing safflower oil or linseed oil. Replacement of 50% of the safflower oil in the diet with linseed oil increased α-linolenic, docosapentaenoic and docosahexaenoic acids in plasma, liver, heart and epididymal fat pad lipids. These results suggest that dietary 18:2ω6 shifts cholesterol from plasma to liver pools followed by redistribution of 20:4ω6 from tissue to plasma pools. This redistribution pattern was not apparent when 18:3ω3 was included in the diet. *Lipids 23,* 847–852 (1988).

Attempts to elucidate mechanisms by which dietary polyunsaturated fatty acids (PUFA) lower blood cholesterol level in man (1–2) and animals (3–7) have led to the hypothesis that dietary PUFA cause redistribution of cholesterol from blood to tissue pools. To test this hypothesis, several studies have examined the effect of dietary PUFA on plasma and tissue cholesterol levels (8–12), using vegetable oils rich in linoleic acid (18:2ω6). Marine oils rich in eicosapentaenoic (20:5ω3) and/or docosahexaenoic acid (22:6ω3) have been shown to be more effective against hypercholesterolemia (12–15). However, one study (16) has shown that both the vegetable oil and fish oil cause similar decreases in cholesterol levels. While vegetable oils rich in ω6 fatty acids have proaggregatory effects in thrombosis (17–19), marine oils rich in very long ω3 fatty acids may account for the enhanced bleeding tendency observed (20–22), due to their antiaggregatory effects. Some studies have shown that vegetable oils have antiaggregatory effects (23,24). However, these studies suggest one thing in common, that the number and arrangement of double bonds within a fatty acid molecule is an important factor for cholesterol-lowering and thrombotic activity. Eicosapentaenoic and docosahexaenoic acids present in marine oils are the desaturated-chain elongated products of α-linolenic (18:3ω3) acid (25). The question of whether 18:3ω3 can affect the plasma and/or tissue cholesterol and fatty acid metabolism has not been given much attention. It is conceivable that 18:3ω3 may have an intermediate effect, in terms of its antiaggregatory and hypocholesterolemic effects, between that of ω6 fatty acids of vegetable oils and very long-chain ω3 fatty acids of marine oils.

The present experiment was designed to clarify the effect of feeding 18:3ω3 on tissue cholesterol pools and fatty acid composition. Safflower- and linseed-oil-fed rats were used to identify the specificity of the action of 18:2ω6 and 18:3ω3 on the distribution of tissue cholesterol pools and levels of 20:4ω6, in comparison with animals fed a diet high in hydrogenated beef tallow.

MATERIALS AND METHODS

Animals and diets. Male adult Sprague-Dawley rats weighing 200–250 g were allotted to individual stainless steel cages and provided water and laboratory chow for three days before starting the experimental diet treatment. After three days of feeding chow, rats were weighed and divided into three groups of five rats per group in a manner ensuring that the average body wt was similar in each group. Each group of animals was fed a semisynthetic diet enriched with either saturated or linoleic or α-linolenic acid. Semipurified diets containing 20% (w/w) fat (26) were prepared by mixing either 180 g hydrogenated beef tallow plus 20 g safflower oil or 200 g safflower oil or 100 g linseed oil plus 100 g safflower oil (Table 1) per kg of total diet. The fatty acid composition of these diets is illustrated (Table 1). The fat mixture of the hydrogenated beef tallow diet contained 26% palmitic and 41% stearic acid, the safflower oil diet was high in linoleic acid (71.2%), while the linseed oil diet provided a high content of α-linolenic acid at 24.1% (Table 1). Diets were prepared weekly and stored at −20 C. Food and water were available to the animals ad libitum for a feeding period of 28 days.

Analytical procedures. After feeding the experimental diets for four weeks, animals were killed between 0800 and 1000 hr and liver, heart and epididymal fat pads were removed and placed in ice-cold physiological saline. After decapitation, trunk blood was collected in heparinized tubes and the plasma was separated (1500 × *g* for 10 min). For lipid extraction, plasma, livers, hearts and epididymal fat pads from animals fed the fat-supplemented diets were homogenized in chloroform/methanol (2:1, v/v) (27). Aliquots of the total lipid extracts from these tissues were taken for determination of free cholesterol, esterified cholesterol and total cholesterol (28). Free cholesterol and esterified cholesterol from lipid extracts of the epididymal fat pad were separated by thin-layer chromatography (TLC) on Silica Gel G plates using a solvent system composed of petroleum ether/diethyl

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Abbreviations: PUFA, polyunsaturated fatty acids; GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

TABLE 1

Fatty Acid Composition of Experimental Diets (wt % of total fatty acids)

Fatty acid[a,b]	Beef tallow[c]	Safflower oil[c]	Linseed oil[c]
14:0	3.7	0.5	0.3
15:0	0.5	—	—
16:0	26.1	8.1	6.3
16:1ω7	0.3	—	0.1
17:0	1.5	—	—
18:0	47.1	2.7	3.8
18:1ω9	5.7	12.4	18.8
18:1ω7	0.7	0.7	0.6
18:2ω6	9.0	71.2	43.3
18:3ω6	0.1	1.2	0.2
18:3ω3	0.1	0.2	24.1
18:4ω3	—	—	1.0
Others	0.2	0.3	0.3
ΣSaturated	79.9	11.6	10.8
ΣMonounsaturated	6.7	13.2	19.8
Σω6	9.2	72.2	44.2
Σω3	0.1	0.2	25.2

[a]Fatty acids are designated by the chain length, number of double bonds and the position of the first double bond from the methyl end of the molecule.

[b]Only major individual fatty acids are reported.

[c]Fats were added to the basal diet in the following proportions: 180 g hydrogenated beef tallow plus 20 g safflower oil (beef tallow diet); 200 g safflower oil (safflower oil diet); and 100 g linseed oil plus 100 g safflower oil (linseed oil diet) per kg of diet. The basal diet contained the following components (g/kg diet): casein, 270; starch, 200; glucose, 207; nonnutritive cellulose, 50; vitamin mix, 10; mineral mix, 50; choline, 2.75; L-methionine, 6.25; as described elsewhere (23).

ether/acetic acid (80:20:1, v/v/v), extracted and then assayed individually (28).

Lipid extracts were converted to fatty acid methyl esters using BF_3-methanol (14%, w/w) reagent (29). Fatty acid methyl esters were separated and quantified by automated gas-liquid chromatography (GLC) (Varian, Model-6000; 30), using a 20-m fused-silica capillary column (BP-20, S.G.E. Pty. Ltd., Melbourne, Australia). Helium was used as the carrier gas. Injector and detector temperatures were maintained at 250 C. The column temperature was programmed from 130 C to 225 C, at a rate of 20 C/min up to 175 C, and then at a rate of 5 C/min up to 225 C. These chromatographic conditions separated all major positional and geometric isomers for fatty acids from 12 to 24 carbons in chain length. Fatty acid methyl ester peaks were identified by injecting authentic standard mixtures of fatty acid methyl esters.

STATISTICAL ANALYSIS

All data are presented as the mean ± standard deviation. The effect of dietary fat supplements was analyzed using analysis of variance procedures and the effect of individual diets was compared for statistical significance ($p < 0.05$) using a Duncan's multiple range test (31).

RESULTS

The average body wt, liver wt, liver wt to body wt percentages, heart wt and amount of food consumed per day for the animals fed the fat-supplemented diets for a four wk period are presented (Table 2). Rats fed the linseed oil diet had significantly higher body wt compared with those fed the hydrogenated beef tallow diet. Animals consumed similar amounts of food per day irrespective of the fat supplement. The wet wt of the livers, liver wt to body wt ratios and heart wt were also unaffected by the dietary fatty acid composition (Table 2).

Feeding the safflower oil diet for 28 days reduced plasma cholesterol content compared with control animals fed beef tallow (Table 3). This reduction was detected in the esterified-cholesterol content while the unesterified-cholesterol content was increased in the plasma of rats fed the safflower oil diet. Feeding the linseed oil diet was more effective in lowering the plasma cholesterol level (Table 3). Total cholesterol content of liver tissue was significantly increased following feeding of the safflower oil diet. This increase was due to accumulation of both free and esterified cholesterol in the liver. Replacement of 50% of the safflower oil by linseed oil reduced accumulation of cholesterol in liver tissue. Cholesterol levels in heart and epididymal fat pads were unchanged by dietary treatments. Neither the free- nor the esterified-cholesterol content of heart or the epididymal fat pad was affected by changes in the dietary fat composition (Table 3).

Essential fatty acid (18:2ω6 and 20:4ω6) content of plasma was elevated by feeding the safflower oil diet, with an accompanied decrease in 16:0, 18:0 and 18:1 content when compared with animals fed diets containing beef tallow (Table 4, Fig. 1). Feeding the linseed oil diet resulted in accumulation of 18:3ω3, 22:5ω3 and 22:6ω3 in the plasma lipids, but failed to alter the 20:4ω6 content. In liver tissue, both the safflower oil and the linseed oil diets lowered the 20:4ω6 content (Table 4, Fig. 1) to the

TABLE 2

Effect of Dietary Fat Treatments on the Liver Weight, Body Weight, Heart Weight, Liver Weight to Body Weight Ratios and Food Consumption[a]

Diet	Body wt (g)	Liver wt (g)	LW/BW (%)	Heart wt (g)	Food consumed (g/day)
Beef tallow	370 ± 14	14.1 ± 0.6	3.8 ± 0.2	1.1 ± 0.1	21.8 ± 1.9
Safflower oil	401 ± 20	14.5 ± 0.7	3.6 ± 0.2	1.2 ± 0.1	20.4 ± 1.8
Linseed oil	421 ± 22[b]	16.1 ± 1.5	3.8 ± 0.2	1.2 ± 0.1	22.9 ± 1.8

[a]Values given are the mean ± standard deviation of 5 rats.

[b]Significantly different from the beef tallow-fed animals at $p < 0.05$.

TABLE 3

Effect of Dietary Fat Treatment on the Cholesterol Content of Rat Plasma, Liver, Heart and Epididymal Fat Pad[a]

Tissue	Cholesterol fraction	Beef tallow	Safflower oil	Linseed oil
Plasma	Free	29.8 ± 2.5	34.2 ± 1.2[b]	30.0 ± 3.0
(mg/100 ml)	Esterified	39.4 ± 2.0	28.1 ± 2.9[c]	25.2 ± 3.4[c]
	Total	69.2 ± 1.6	62.1 ± 1.9[b]	55.2 ± 1.7[c]
Liver	Free	130 ± 11	156 ± 7[b]	137 ± 7
(mg/100 g)	Esterified	70 ± 6	95 ± 12[b]	76 ± 14
	Total	200 ± 15	251 ± 15[b]	214 ± 12
Heart	Free	108 ± 7	107 ± 4	117 ± 11
(mg/100 g)	Esterified	26 ± 1	26 ± 3	29 ± 2
	Total	135 ± 7	133 ± 6	146 ± 14
Adipose tissue	Free	133 ± 3	135 ± 4	136 ± 6
(mg/100 g)	Esterified	32 ± 2	31 ± 2	32 ± 4
	Total	165 ± 3	166 ± 3	168 ± 6

[a]Values are the mean ± standard deviation for 5 animals.

[b]Significantly different from beef tallow-fed animals at $p < 0.05$.

[c]Significantly different from beef tallow-fed animals at $p < 0.01$.

TABLE 4

Effect of Dietary Fat Treatments on the Fatty Acid Composition of Rat Plasma and Liver Total Lipids (wt % of total fatty acids)[a]

	Plasma			Liver		
Fatty acid	Beef tallow	Safflower oil	Linseed oil	Beef tallow	Safflower oil	Linseed oil
14:0	2.3 ± 0.5[a]	0.4 ± 0.1[b]	0.3 ± 0.0[b]	—	—	—
16:0	22.3 ± 1.0[a]	15.5 ± 1.1[b]	13.1 ± 1.1[b]	15.4 ± 0.8	14.9 ± 0.8	13.5 ± 0.7
16:1ω7	1.6 ± 0.5[a]	0.5 ± 0.1[b]	0.3 ± 0.1[c]	1.2 ± 0.3[a]	0.4 ± 0.1[b]	0.3 ± 0.0[b]
17:0	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	0.6 ± 0.1[a]	0.2 ± 0.0[b]	0.3 ± 0.0[c]
18:0	21.8 ± 0.9[a]	13.0 ± 1.4[b]	14.3 ± 2.6[b]	25.7 ± 0.8[a]	14.7 ± 1.8[b]	19.4 ± 3.9[c]
18:1ω9	15.2 ± 1.4[a]	8.0 ± 0.8[b]	10.2 ± 1.8[b]	8.2 ± 0.5	6.9 ± 0.6	7.7 ± 1.9
18:1ω7	2.2 ± 0.3[a]	1.8 ± 0.4[a]	1.3 ± 0.1[b]	2.5 ± 0.4	2.2 ± 0.4	1.8 ± 0.1
18:2ω6	18.5 ± 1.3[a]	40.3 ± 4.1[b]	32.8 ± 2.8[c]	11.2 ± 0.8[a]	32.4 ± 3.6[b]	23.3 ± 4.0[b]
18:3ω6	0.2 ± 0.1[a]	0.3 ± 0.1[a]	0.1 ± 0.0[b]	0.2 ± 0.0[a]	0.5 ± 0.1[b]	0.2 ± 0.1[a]
18:3ω3	T[b,a]	—	8.3 ± 1.9[b]	0.3 ± 0.2[a]	0.1 ± 0.0[a]	3.8 ± 1.4[b]
20:1ω9	0.2 ± 0.1	—	—	0.1 ± 0.0[a]	0.2 ± 0.0[b]	0.2 ± 0.1[b]
20:2ω6	0.1 ± 0.0[a]	0.9 ± 0.1[b]	0.4 ± 0.1[c]	0.3 ± 0.1[a]	1.5 ± 0.2[b]	0.8 ± 0.2[c]
20:3ω9	0.2 ± 0.0[a]	0.2 ± 0.0[a]	0.1 ± 0.0[b]	—	—	—
20:3ω6	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
20:4ω6	11.4 ± 0.4[a]	14.7 ± 1.2[b]	11.8 ± 2.8[a,b]	25.0 ± 1.4[a]	18.0 ± 1.5[b]	18.4 ± 2.4[b]
20:5ω3	—	—	—	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
22:4ω6	—	0.7 ± 0.2[a]	0.1 ± 0.0[b]	0.4 ± 0.1[a]	1.0 ± 0.2[b]	0.2 ± 0.0[c]
22:5ω6	0.6 ± 0.2[a]	0.9 ± 0.2[a]	T	1.3 ± 0.2	1.6 ± 0.2	—
22:5ω3	0.3 ± 0.2[a]	0.2 ± 0.1[a]	0.6 ± 0.1[b]	0.1 ± 0.0[a]	—	0.9 ± 0.1[b]
22:6ω3	0.9 ± 0.1[a]	0.7 ± 0.2[a]	2.1 ± 0.4[b]	3.1 ± 0.2[a]	1.6 ± 0.2[b]	4.3 ± 0.6[c]
ΣSat.	47.7 ± 1.4	30.2 ± 2.8	28.5 ± 3.8	42.7 ± 0.9	30.6 ± 2.5	33.7 ± 4.3
ΣMUFA[c]	19.6 ± 1.6	10.8 ± 1.2	12.5 ± 1.8	12.7 ± 0.9	10.5 ± 0.5	11.2 ± 1.9
Σω6	31.2 ± 1.8	57.9 ± 3.2	46.8 ± 1.2	38.6 ± 1.1	55.1 ± 3.0	43.2 ± 1.4
Σω3	1.5 ± 0.3	1.1 ± 0.2	11.4 ± 1.5	3.6 ± 0.3	1.8 ± 0.2	9.4 ± 1.0

[a]Values given are mean ± standard deviation of 5 separate determinations (n = 5). Values without a common superscript are significantly different at $p < 0.05$.

[b]Trace amount (<0.1).

[c]Monounsaturated fatty acid.

same extent (ca. 28% compared with the group fed beef tallow). Consumption of the safflower oil diet increased deposition of 18:2ω6, 20:2ω6 and 22:4ω6 and depletion of 22:5ω3 and 22:6ω3 in the liver lipids. On the other hand, 18:3ω3, 22:5ω3 and 22:6ω3 in the liver tissue increased following the feeding of the linseed oil diet (Table 4).

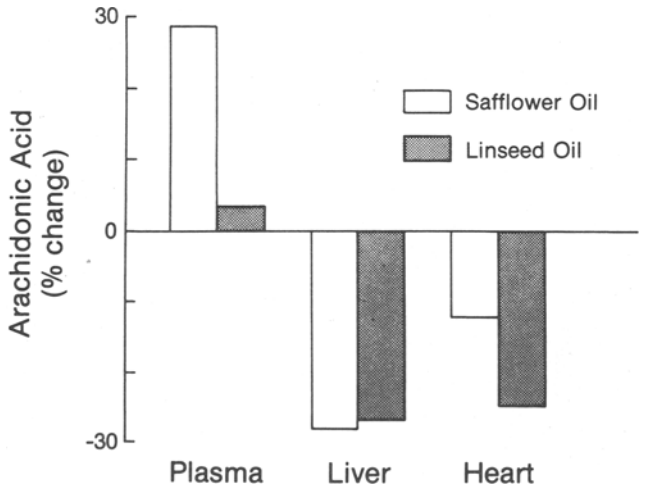

FIG. 1. Percentage change in the arachidonic acid content of plasma, liver and heart lipids compared with the hydrogenated beef tallow group.

Ingestion of the safflower oil diet increased 18:2ω6 and 22:5ω6 content in cardiac lipids and was accompanied by decreased 20:4ω6 and 22:6ω3 content (Table 5). Feeding the linseed oil diet decreased the 20:4ω6 levels in the heart more effectively than observed for the safflower oil diet (12.2% vs 24.9%, respectively; Fig. 1). The linseed oil diet also increased 18:3ω3, 22:5ω3 and 22:6ω3 in heart lipids (Table 5). The fatty acid composition of the epididymal fat pad resembled that of the dietary fat treatment, e.g., the safflower oil diet increased 18:2ω6 content, whereas the linseed oil diet elevated 18:2ω6 and 18:3ω3 content at the expense of saturated (14:0, 16:0 and 18:0) and monounsaturated (16:1 and 18:1) fatty acids (Table 5).

DISCUSSION

The results suggest that dietary 18:2ω6 and 18:3ω3 act differentially to alter lipid metabolism of rat tissues. Animals fed the diet containing linseed oil grew faster than those fed the beef tallow diet (Table 2). The amount of food consumed per day, although not statistically different ($p > 0.05$), was somewhat higher in the linseed diet fed group. Therefore, it is conceivable that the increase in body wt after linseed oil consumption may be due to increased food intake. Liver wt, heart wt, and liver wt to body wt ratios, however, remained unchanged by the dietary fat treatments (Table 2). Therefore, the changes observed in cholesterol content and fatty acid composition of tissues apparently are a result of dietary fat treatment and do not stem from differences in growth rate.

TABLE 5

Effect of Dietary Fat Treatments on the Fatty Acid Composition of Rat Heart and Epididymal Fat Pads (wt % of total fatty acids)[a]

	Heart			Epididymal fat pad		
Fatty acid	Beef tallow	Safflower oil	Linseed oil	Beef tallow	Safflower oil	Linseed oil
14:0	1.1 ± 0.1[a]	0.6 ± 0.2[b]	0.5 ± 0.2[b]	3.7 ± 0.3[a]	0.7 ± 0.4[b]	1.0 ± 0.3[b]
16:0	12.5 ± 0.6[a]	10.0 ± 0.6[b]	9.5 ± 0.7[b]	24.9 ± 0.6[a]	13.6 ± 0.9[b]	14.5 ± 1.3[b]
16:1ω7	0.7 ± 0.1	0.5 ± 0.1	0.4 ± 0.2	4.7 ± 0.3[a]	1.6 ± 0.2[b]	2.1 ± 0.4[b]
17:0	0.4 ± 0.0[a]	0.2 ± 0.0[b]	0.2 ± 0.0[b]	0.7 ± 0.0[a]	0.1 ± 0.1[b]	0.2 ± 0.0[c]
18:0	24.9 ± 0.8	23.4 ± 0.7	24.3 ± 0.8	10.7 ± 0.6[a]	3.0 ± 0.6[b]	4.8 ± 1.0[b]
18:1ω9	3.5 ± 0.2	3.1 ± 0.1	4.3 ± 0.8	25.6 ± 1.7[a]	16.4 ± 0.6[b]	22.0 ± 0.9[a]
18:1ω7	3.1 ± 0.2[a]	2.6 ± 0.3[a,b]	2.4 ± 0.1[b]	3.3 ± 0.2[a]	1.7 ± 0.2[b]	1.7 ± 0.4[b]
18:2ω6	17.1 ± 1.0[a]	26.6 ± 1.0[b]	23.6 ± 1.9[c]	21.2 ± 1.5[a]	58.1 ± 2.0[b]	35.1 ± 2.2[c]
18:3ω6	—	—	—	—	—	—
18:3ω3	—	—	1.8 ± 0.5	0.8 ± 0.2[a]	0.6 ± 0.3[a]	14.3 ± 1.3[b]
20:3ω6	—	0.3 ± 0.1	—	—	—	—
20:4ω6	23.7 ± 0.6[a]	20.8 ± 0.5[b]	17.8 ± 0.4[c]	—	—	—
22:4ω6	0.3 ± 0.3	0.2 ± 0.0	0.4 ± 0.1	—	—	—
22:5ω6	1.9 ± 0.4[a]	4.7 ± 0.5[b]	T[b,c]	—	—	—
22:5ω3	0.9 ± 0.1[a]	0.5 ± 0.2[b]	2.9 ± 0.2[c]	—	—	—
22:6ω3	7.8 ± 0.4[a]	4.8 ± 0.6[b]	9.7 ± 1.1[c]	—	—	—
Others	—	—	—	0.9 ± 0.3[a]	2.1 ± 0.4[b]	1.6 ± 0.3[b]
ΣSat.	39.5 ± 0.7	34.4 ± 0.5	35.2 ± 1.1	40.6 ± 0.7	17.7 ± 1.6	21.0 ± 2.0
ΣMUFA[c]	8.1 ± 0.5	6.9 ± 0.3	7.9 ± 0.9	34.0 ± 1.6	18.4 ± 1.2	25.6 ± 1.1
Σω6	43.4 ± 0.8	53.1 ± 0.8	42.5 ± 1.3	21.9 ± 1.5	59.9 ± 2.4	35.1 ± 2.2
Σω3	8.9 ± 0.8	5.3 ± 0.5	14.5 ± 1.2	0.8 ± 0.2	0.6 ± 0.3	14.3 ± 1.3

[a]Values given are mean ± standard deviation of 5 separate determinations (n = 5). Values without a common superscript are significantly different at $p < 0.05$.

[b]Trace amount (<0.1).

[c]Monounsaturated fatty acid.

The reduction in plasma cholesterol level following consumption of the safflower oil diet accompanied by increased liver cholesterol content is in agreement with previous observations (8–12) that suggest PUFA of the ω6 family increase cholesterol accumulation in liver relative to feeding diets high in saturated fatty acids. Both free- and cholesterol-ester content increased following feeding of the safflower oil diet, but this increase was more pronounced in the cholesteryl-ester fraction. This observation is consistent with the fact that diets rich in linoleic acid (18:2ω6) accelerate esterification of cholesterol by acyl coenzyme A:cholesterol acyltransferase (EC 2.3.1.26) activity in the rat liver microsomes (32). This increased esterification of cholesterol following feeding of the safflower oil diet, in turn, may increase the capacity of hepatic cells to take up more free cholesterol from circulating levels in the plasma (33). Replacement of 50% of safflower oil with linseed oil restricted deposition of cholesteryl esters in the liver and concomitantly lowered the plasma cholesterol level to an even greater extent than the safflower oil diet. In the light of these results, the hypothesis that dietary PUFA shift plasma cholesterol to tissue cholesterol pools cannot be generalized for all types of PUFA and may be restricted to ω6 fatty acids only. The cholesterol levels in heart and adipose tissue of rats, however, remained unaltered by the composition of the fat fed.

Consumption of the safflower oil diet resulted in a significant increase in plasma 20:4ω6 level, while in liver and heart lipid, the concentration of 20:4ω6 was significantly decreased in comparison with animals fed the diet high in beef tallow (Tables 4 and 5, Fig. 1). Increase in plasma 20:4ω6 content may be due to increased synthesis in the liver microsomes via desaturation and chain elongation of 18:2ω6 (34). However, this seems unlikely as the 20:4ω6 level in the liver decreased when rats were fed the safflower oil diet. Increased 20:4ω6 content in plasma lipids with concomitant depletion of 20:4ω6 in liver and heart tissue for animals fed the safflower oil diet treatment may be taken as evidence for shift of tissue 20:4ω6 to plasma pools. Some studies have reported either no change (35), or even a decrease (36), in liver microsomal Δ^6-desaturase activity, a rate-limiting enzyme in the 20:4ω6 biosynthetic pathway, following feeding of a diet rich in linoleic acid. Previous evidence indicates transfer of cholesterol from plasma to the liver pools with participation of liver phosphatidylcholine containing 20:4ω6 in the SN-2 position (37). The present paper demonstrates that this process of transfer of plasma cholesterol to liver pools and liver 20:4ω6 transport to plasma pools occurs at a faster rate when rats are fed an 18:2ω6 enriched diet. Inclusion of 18:3ω3 in the diet appears to slow this cycle by depleting the liver 20:4ω6 pool via inhibition of 20:4ω6 biosynthesis. In this context, the diet rich in 18:3ω3 did not change the 20:4ω6 content of plasma, depleted the 20:4ω6 level in the liver to the same extent as observed for animals fed the safflower oil diet, and depleted the 20:4ω6 level to an even greater extent in heart lipids. α-Linolenic present in the linseed oil diet competes with 18:2ω6 for Δ^6-desaturation, thus limiting synthesis of 20:4ω6 from 18:2ω6 (38). It is also conceivable that some of the tissue 20:4ω6 may shift to plasma pools for maintenance of circulating levels of 20:4ω6. These observations are further supported by the observation that an increase in the desaturated-chain elongated products of 18:3ω3 metabolism, i.e., 22:5ω3 and 22:6ω3, occurs in plasma, liver and heart lipids of rats fed the linseed oil diet. Interestingly, 20:5ω3 did not accumulate in any of the tissues examined even after feeding a high dietary load of 18:3ω3. In an earlier study, we noticed an increase in plasma and tissue 20:5 levels (39) following the feeding of 18:3ω3 rich diets. This contradiction may be due to the fact that the linseed oil diet in the present study contained a high level of 18:2ω6 in addition to a high 18:3ω3 content, whereas in the previous study, 18:3ω3 was fed to the rats along with saturated and monounsaturated fatty acids (39). In this regard, the balance between ω6 and ω3 fatty acids recently has been shown to be an important factor for modification of desaturase enzymes responsible for synthesis of 20:5ω3 and 22:6ω3 from 18:3ω3 (40). The decrease in monounsaturated fatty acids (16:1ω7 and/or 18:1ω9) in rat tissues by the diets containing high levels of 18:2ω6 and/or 18:3ω3 is consistent with the fact that PUFA inhibit Δ^9-desaturase activity (34,35).

The present study demonstrates that dietary 18:3ω3 is more potent than 18:2ω6 in lowering plasma cholesterol level and does not result in accumulation of cholesterol in liver tissue. However, 18:3ω3 appears to be relatively less effective for reducing cholesterol level than 20:5ω3 and/or 22:6ω3 present in fish oils (12–15). Feeding diets high in linseed oil for 28 days did not lower plasma 20:4ω6 level, but depleted tissue levels. Longer-term use of 18:3ω3 supplements may lower plasma 20:4ω6 level and, thus, may show antiaggregatory effects (19). Consumption of fish oils rich in 20:5ω3 and/or 22:6ω3 lowers plasma, platelet and tissue 20:4ω6 content more effectively even after a short-term feeding (41,42), which may account for the enhanced bleeding time observed after such diet treatments. Therefore, 18:3ω3 appears to have an effective intermediate in reducing cholesterol levels and, perhaps, platelet aggregation between that of 18:2ω6 and 20:5ω3. As ω6 PUFA shift cholesterol from blood to the liver pools, but do not lower total body cholesterol, they may not be as beneficial against cardiovascular diseases as they currently are thought to be. However, α-linolenic acid from linseed oil appears to lower plasma cholesterol without accumulation of cholesterol in the liver tissue. Dietary PUFA have also been shown to have different lipid-lowering effects in animals and humans (43,44), e.g., fish oil lowers the circulating cholesterol level in the rat (43), but not in the human (44). Therefore, the effect of α-linolenic acid should be examined in human subjects with appropriate controls, before any further conclusion can be drawn.

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REFERENCES

1. Spritz, N., Ahrens, E.H., Jr., and Grundy, S. (1965) *J. Clin. Invest. 44,* 1482–1493.
2. Grundy, S.M., and Ahrens, E.H., Jr. (1970) *J. Clin. Invest. 49,* 1135–1152.

3. Avigan, J., and Steinberg, D. (1958) *Proc. Soc. Exp. Biol. Med. 97,* 814-816.
4. Bloomfield, D.K. (1964) *J. Lab. Clin. Med. 64,* 613-623.
5. Bieberdorf, F.A., and Wilson, J.D. (1965) *J. Clin. Invest. 44,* 1834-1844.
6. Corey, J.E., Nicolosi, R.J., and Hayes, K.C. (1976) *Exp. Mol. Pathol. 25,* 311-321.
7. Krause, B.R., and Hartman, A.D. (1984) *J. Lipid Res. 25,* 97-110.
8. Peifer, J.J. (1966) *J. Nutr. 88,* 351-358.
9. Gerson, T., Shorland, F.B., and Adams, Y. (1961) *Biochem. J. 81,* 584-590.
10. Huang, Y.S., Manku, M.S., and Horrobin, D.F. (1984) *Lipids 19,* 664-672.
11. Garg, M.L., Snoswell, A.M., and Sabine, J.R. (1985) *Nutr. Rep. Int. 32,* 117-127.
12. Balasubramaniam, S., Simons, L.A., Chang, S., and Hickie, J.B. (1985) *J. Lipid Res. 26,* 684-689.
13. Kobatake, Y., Kuroda, K., Jinnouchi, H., Nishide, E., and Innami, S. (1984) *J. Nutr. Sci. Vitaminol. 30,* 357-372.
14. Nestel, P.J. (1986) *Am. J. Clin. Nutr. 43,* 752-757.
15. Herold, P.M., and Kinsella, J.E. (1986) *Am. J. Clin. Nutr. 43,* 566-598.
16. Harris, W.S., Connor, W.E., and McMurry, M.P. (1983) *Metabolism 32,* 179-184.
17. Croft, K.D., Beilin, L.J., Vandongen, R., and Mathews, E. (1984) *Biochim. Biophys. Acta 795,* 196-207.
18. Hamberg, M., Svensson, J., and Samuelsson, B. (1975) *Proc. Natl. Acad. Sci. USA 72,* 2994-2998.
19. Marshall, L.A., and Johnston, P.V. (1982) *Lipids 17,* 905-913.
20. Ahmed, A.A., and Holub, B.J. (1984) *Lipids 19,* 617-624.
21. Sanders, T.A.B., Vickers, M., and Haines, A.P. (1981) *Clin. Sci. 61,* 317-324.
22. Sanders, T.A.B., Naismith, D.J., Haines, A.P., and Vickers, M. (1980) *Lancet 1,* 1189.
23. McGregor, L., Morazain, R., and Renaud, S. (1980) *Thrombosis Res. 20,* 499-507.
24. Fleischman, A., Bierenbaum, M.L., Justice, D., Stier, A., Sullivan, A., and Fleischman, M. (1975) *Amer. J. Clin. Nutr. 28,* 601-605.
25. Dyerberg, J. (1986) *Nutr. Rev. 44,* 125-134.
26. Clandinin, M.T., and Yamashiro, S. (1982) *J. Nutr. 112,* 825-828.
27. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
28. Sale, F.O., Marchesini, S., Fishman, P.H., and Berra, B. (1984) *Anal. Biochem. 142,* 347-350.
29. Metcalfe, L.D., and Schmitz, A.A. (1961) *Anal. Chem. 33,* 363-364.
30. Hargreaves, K.M., and Clandinin, M.T. (1987) *Biochim. Biophys. Acta 918,* 97-105.
31. Steel, R.G.D., and Torrie, J.H. (1980) *Principles and Procedures of Statistics, A Biometrical Approach,* McGraw-Hill Book Co., Inc., New York, NY.
32. Spector, A.A., Kaduce, T.L., and Dane, R.W. (1980) *J. Lipid Res. 21,* 169-179.
33. Reiber, H. (1978) *Biochim. Biophys. Acta 512,* 72-83.
34. Pugh, E.L., and Kates, M. (1984) *Lipids 19,* 48-55.
35. Mahfouz, M.M., Smith, T.L., and Kummerow, F.A. (1984) *Lipids 19,* 214-222.
36. Brenner, R.R. (1981) *Prog. Lipid Res. 20,* 41-47.
37. Holub, B.J., and Kuksis, A. (1971) *Can. J. Biochem. 49,* 1005-1011.
38. Brenner, R.R. (1974) *Mol. Cell. Biochem. 3,* 41-52.
39. Garg, M.L., Sebokova, E., Thomson, A.B.R., and Clandinin, M.T. (1988) *Biochem. J. 249,* 351-356.
40. Cook, H.W., and Spence, M.W. (1987) *Lipids 22,* 613-619.
41. Dyerberg, J., and Jorgensen, K.A. (1982) *Prog. Lipid Res. 21,* 255-269.
42. Holmer, G., and Beare-Rogers, J.L. (1985) *Nutr. Res. 5,* 1011-1014.
43. Balasubramaniam, S., Simons, L.A., Chang, S., and Hickie, J.B. (1985) *J. Lipid Res. 26,* 684-689.
44. Demke, D.M., Peters, G.R., Linet, O.I., Metzler, C.M., and Klott, K.A. (1988) *Atherosclerosis 70,* 73-80.

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Effects of Estrogen-induced Hyperlipidemia on the Erythrocyte Membrane in Chicks

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The effects of estrogen-induced hyperlipidemia on plasma lipid peroxidation, fatty acid composition and osmotic fragility of erythrocytes in chickens were studied. Young male chickens implanted with estrogen for three wk developed a marked hyperlipidemia. Plasma levels of triglyceride, cholesterol and phospholipid were elevated 68-, four- and 24-fold, respectively, over controls. There was also a two-fold increase in plasma lipid peroxidation measured by the thiobarbituric acid test. Vitamin E supplement (1,000 IU/kg diet) reduced the plasma lipid peroxidation to the control level, but had no effect on the plasma lipid content. Estrogen-induced hyperlipidemia resulted in changes in the fatty acid composition of membrane lipids of erythrocytes. The major changes were an increase in oleic acid from 10.0% to 14.2% and a decrease in linoleic acid from 31.3% to 26.0%. The erythrocytes with an altered membrane fatty acid composition were found to have an increased osmotic fragility. It was apparent that there was a direct correlation between the oleic acid content and the osmotic fragility of erythrocytes.
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It is well-established that the physical state of membrane lipids and, thus, indirectly their composition, are important in the functioning of membranes (1). Alterations in membrane lipid composition induced by diet affect glucagon-stimulated adenylate cyclase activity in liver membranes (2) and Ca^{2+}-ATPase activity in rat erythrocytes (3). Changes in the erythrocyte fatty acid composition also result in an increased osmotic fragility (4). Erythrocyte aging is also accompanied by increased osmotic fragility, increased density, accumulation of fluorescent chromolipids and the formation of high molecular-wt proteins (5). These changes can be induced in vitro by the addition of the secondary peroxidation product, malondialdehyde (5–7). Peroxidation products are found in the blood of restricted ovulator chickens (8). These chickens have a greatly elevated blood lipid level (9) and develop atherosclerosis at an early age (10).

It was our purpose here to investigate the effect of hyperlipidemia on the erythrocyte membrane. To do this, we used an alternative model to the restricted ovulator chicken. The genetic defect in restricted ovulator chickens causes a rise in estrogen levels and this, in turn, seems to be responsible for the alteration in lipid metabolism (11). Studies in our laboratory have shown that implantation of estrogen in young male chickens resulted in a hyperlipidemia similar to that seen in restricted ovulator chickens. The advantages of this model are that the use of male chickens eliminates the cycling of blood lipid levels seen in laying hens, and it is not necessary to wait for pubescence to begin an experiment.

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Abbreviation: TBARS, thiobarbituric acid reacting substance.

MATERIALS AND METHODS

Animals. Male chickens (New Hampshire male × Columbian female), seven weeks old, were used in these experiments. The birds were housed individually in wire cages under a constant lighting program (09:00–21:00 hour). Chicks were fed ad libitum a pullet starter-grain diet (Table 1). In the vitamin E-supplemented group, vitamin E was added to the feed at the level of 1,000 IU/kg of diet.

Preparation of estrogen implants. Estrogen tubes were prepared by the method of Smith et al. (12). Briefly, polymethylsiloxan tubing (1.5 mm i.d. × 3.13 mm o.d.) was cut into 6-cm lengths, filled with approximately 50 mg of β-estradiol dipropionate (Sigma Chemical Co., St. Louis, MO), and both ends of tubes were sealed with Medical Adhesive Silicon Type A. Prior to implantation, the tubes were incubated in 0.01 M phosphate buffered saline (pH 7.4) for 48 hr at 37 C. Under light ether anesthesia, the necks of the chickens were incised (0.5–1.0 cm) with a surgical knife and tubes were inserted through the incisions. Incisions were closed with an Auto Suture skin stapler.

Analysis of blood lipids. At the end of the three-week treatment period and after an overnight fast, blood was drawn from the wing vein into heparinized tubes and centrifuged at 1,000 × *g* for 20 min at 4 C. The plasma was removed and the level of plasma total cholesterol was measured enzymatically (13), using A-Gent cholesterol reagent (Abbott Laboratories, N. Chicago, IL). The level of plasma triglycerides was measured by a colorimetric

TABLE 1

Composition of Diet

Ingredient	%
Ground yellow corn	61.75
Dehulled soybean meal	24.05
Wheat bran	5.00
Meat and bone meal	4.00
Alfalfa meal	2.00
Dicalcium phosphate	2.00
Limestone	0.60
Salt	0.30
Trace mineral mix[a]	0.05
Vitamin mix[b]	0.25
Calculated composition:	
Metabolizable energy (Kcal/kg)	2.882.0
Crude protein (%)	20.2

[a]Trace mineral mix provided (per kg diet): 75 mg manganese, 75 mg iron, 75 mg zinc, 5 mg copper, 0.75 mg iodine and 0.1 mg selenium.
[b]Vitamin mix provided (per kg diet): 4400 USPU vitamin A, 1000 USPU vitamin D_3, 11 IU vitamin E, 4.4 mg riboflavin, 10 mg d-pantothenic acid, 22 mg niacin, 11 μg vitamin B_{12} and 2.3 mg menadion sodium bisulfate.

Hantzsch condensation method of Foster and Dunn (14). The phospholipid content was determined by measuring the phosphorus content of plasma total lipid extract after perchloric acid digestion (15), and multiplying by 25.5.

Determination of thiobarbituric acid reacting substances (TBARS). TBARS were determined using the flourometric assay of Yagi (16). Twenty μl of plasma were diluted with 4.0 ml of 0.08 N H_2SO_4, followed by addition of 0.5 ml of 10% phosphotungstic acid. After sitting at room temperature for 5 min, the samples were centrifuged at 1,000 × *g* for 10 min. The supernate was removed and the pellet was resuspended with the above solution and centrifuged again. The washed pellet was then suspended in 4.0 ml of distilled water and 1.0 ml of TBA reagent (thiobarbituric acid/glacial acetic acid, 1:1, v/v). After incubation for 1 hr at 95 C, the tubes were cooled under tap water and 5.0 ml of n-butanol added. The tubes were shaken and centrifuged at 1,000 × *g* for 15 min. The butanol layer was taken for fluorometric measurement at 553 nm with excitation at 515 nm. Standards of 0.5 nmol tetramethoxypropane in 4.0 ml of distilled water were incubated with 1.0 ml of TBA reagent, extracted and read at the same time as the samples. The amount of TBARS was determined by the equation (Iu/Is) × 25 = nmol malondialdehyde/ml of plasma, where Iu = fluorescence intensity of the sample, and Is = fluorescence intensity of the standard.

Preparation of erythrocyte membrane. After removal of plasma, the sedimented erythrocytes were resuspended with isotonic Tris buffer (310 imOsM), pH 7.6, by gentle mixing and centrifuged at 1,000 × *g* for 20 min. The supernate was removed by careful suction and the washing procedure was repeated twice more. The washed erythrocytes were resuspended in the same isotonic Tris buffer to an approximate hematocrit of 50%, and the erythrocyte membranes were then prepared by osmotic lysis with 20 imOsM Tris buffer, according to the method of Hanahan and Ekholm (17).

Analysis of fatty acid composition. Total lipids were extracted from the erythrocyte membrane with 20 volumes of chloroform/methanol (2:1, v/v), according to the method of Folch et al. (18). Butylated hydroxy toluene was added as antioxidant to the lipid-extracting solvent (0.001%). The fatty acid methyl esters of total lipid extracts were prepared with 14% borontrifluoride in methanol at 90 C for 2 hr, by the method of Morrison and Smith (19). A Hewlett-Packard Model 5790 gas chromatograph with dual flame ionization detectors and split capillary injector was used for analysis of fatty acid methyl esters. The capillary column was a highly polar fused silica SP-2330 column (30 m × 0.25 mm i.d., 0.20 μm film thickness). The oven temperature was programmed from 190–210 C at 2 C/min. The split ratio at 190 C was 1:80 with hydrogen carrier at a flow rate of 1.2 ml/min and injector and detector temperatures were 250 C, respectively. The identification of the fatty acid methyl esters was based on retention times relative to methyl stearate and comparison of authentic standard methyl ester mixtures (NuChek Prep., Elysian, MN).

Osmotic fragility test. Buffered saline, pH 7.4 (154 mM NaCl, 85 mM Na_2HPO_4 and 16 mM NaH_2PO_4 $2H_2O$) was prepared and diluted into a series of different concentrations of saline, and the osmotic fragility test was carried out, as described by Dacie and Lewis (20). Briefly, to each test tube containing 5 ml of buffer, 50 μl of whole heparinized blood was added and mixed immediately. After standing for 30 min at room temperature, the samples were centrifuged at 500 × *g* for 5 min, and the supernate was read in a spectrophotometer at 545 nm. The degree of hemolysis is expressed in percentage, where 100% represents full hemolysis.

Data were analyzed by the difference between means, and statistical significance was based on Duncan's new multiple-range test (21).

RESULTS AND DISCUSSION

Chickens with estrogen implants for three wk developed a marked hyperlipidemia as shown in Table 2. The levels of plasma lipids increased 68-fold in triglyceride (60 vs 4,060 mg/dl), four-fold in cholesterol (135 vs 550 mg/dl) and 24-fold in phospholipid (73 vs 1,770 mg/dl), respectively, over the corresponding values of control chickens. The development of endogenous hyperlipidemia appears to be consistent in both humans (22) and avian species (23), as a consequence of estrogen treatment. Compared with controls, the level of plasma lipid peroxidation also increased significantly ($p < 0.05$) in hyperlipidemic chickens with estrogen implants (9.3 vs 20.4 nmol/ml). An increase in plasma lipid peroxidation in the presence of hyperlipidemia has previously been reported in restricted ovulator chickens (8). Vitamin E supplementation (1,000 IU/kg diet) resulted in a two-fold reduction of plasma lipid peroxidation to a level that was statistically no different than that of the controls (10.4 vs 9.3 nmol/ml), but did not alter hyperlipidemia. This finding seems to indicate that an increased amount of vitamin E is required to prevent lipid peroxidation in hyperlipidemic individuals.

The fatty acid composition of erythrocyte membrane lipids in chickens with estrogen implants is shown in Table 3. In control chickens, linoleic acid was the major fatty acid (31.3%), followed by palmitic (18.5%), stearic (18.1%), oleic (10.0%) and arachidonic acid (9.8%). Low levels of docosatetraenoic (2.3%) and docosahexaenoic acids (2.2%) were also present. Estrogen treatment induced an alteration in the fatty acid composition of erythrocyte membrane lipids with major changes in the 18-carbon unsaturated fatty acids. Compared with controls, the estrogen-implanted chickens exhibited an increase in oleic acid (10.0% to 14.2%), with a decrease in linoleic acid (31.3% to 26.0%). There was also a small increase in

TABLE 2

Levels of Plasma Lipids and Lipid Peroxidation in Chickens Implanted With Estrogen for Three Weeks[a]

	Control	Estrogen	Estrogen + vit E
Cholesterol (mg/dl)	135 ± 18[a]	550 ± 150[b]	518 ± 190[b]
Triglyceride (mg/dl)	60 ± 6[a]	4060 ± 703[b]	4430 ± 2070[b]
Phospholipid (mg/dl)	73 ± 20[a]	1770 ± 424[b]	2250 ± 1020[b]
TBARS (nmol/ml)[b]	9.3 ± 1.0[a]	20.4 ± 7.5[b]	10.4 ± 0.9[a]

[a]Mean ± standard deviation for five birds. Values on the same line bearing different superscripts are significantly different ($p < 0.05$).
[b]Thiobarbituric acid reactive substances (TBARS).

palmitic acid (18.5% to 20.7%), but little change occurred in the 20-carbon polyunsaturated fatty acids in chickens with estrogen implants. Estrogen-treated and vitamin E-supplemented chickens had the same fatty acid profile as estrogen-treated chickens, except for stearic acid which was further decreased and oleic acid which was further increased. The enrichment of oleic acid in erythrocyte membrane lipids follows a similar pattern to that seen in the fatty acids of plasma lipids of estrogen-induced hyperlipidemic chickens (24) and of restricted ovulator hens (9). Although the mechanism of changes in the fatty acid composition of avian erythrocytes with hyperlipidemia is not known, the decrease in the amount of linoleic acid relative to oleic and palmitic acids in erythrocyte membrane of the estrogen-treated birds is explainable in terms of the marked stimulation of lipogenesis under estrogenic influence (24). Because the amount of linoleic acid available for incorporation into membrane lipids is constrained by the amount available from the diet, whereas the amounts of oleic and palmitic acid available are markedly increased by endogenous synthesis, these latter fatty acids are more competitive for the acylation of lysophosphatides in the erythrocyte membrane (25).

The osmotic fragilities of erythrocytes from chickens are shown in Figure 1. Compared with controls, the osmotic sensitivity to hypotonic NaCl solutions was increased in estrogen-treated chickens with or without vitamin E supplement. The NaCl concentrations for 50% hemolysis for the estrogen-treated (66.4 mM) and estrogen-treated-plus-vitamin-E-supplemented chickens (68.7 mM) were significantly ($p < 0.05$) higher than that of the control (63.5 mM). However, there was no significant difference between the NaCl concentrations for the estrogen and vitamin E groups. As shown in Figure 2, a strong relationship was noted between the osmotic fragility and the amount of oleic acid present in the erythrocyte membrane. An increased level of oleic acid corresponded to an increased osmotic fragility. Workers in this laboratory previously have noted such an association between the octadecenoate content of rat erythrocytes and their osmotic fragility (4). Phillips et al. have also shown an increase in oleic acid in the fatty acids of aging human erythrocyte (26). Because the low level of linoleic acid present in the erythrocytes should reduce the oxidation potential of the membrane lipids, the inverse relationship between the linoleic acid content of the membrane and osmotic fragility seems paradoxical. One possible explanation might be that the rigidity of the membrane is enhanced by replacement of linoleic acid with oleic acid. An increased osmotic sensitivity to hypotonic treatment has been reported in erythrocytes from essential fatty acid-deficient rats, in which the level of linoleic acid is reduced to nearly half that of the control values (27).

With age, erythrocytes increase in density, accumulate high molecular-wt protein and become less deformable (5). Loss of deformability is a factor involving membrane fluidity and is thought to be a trigger for erythrocyte removal by the spleen. Aging of erythrocytes can be accelerated by genetic factors or drug administration (28). There is mounting evidence that lipid peroxidation plays a crucial role in erythrocyte aging (28,29). The initiation of peroxidation in suspensions of erythrocytes results in all the changes typical of naturally aged erythrocytes (29).

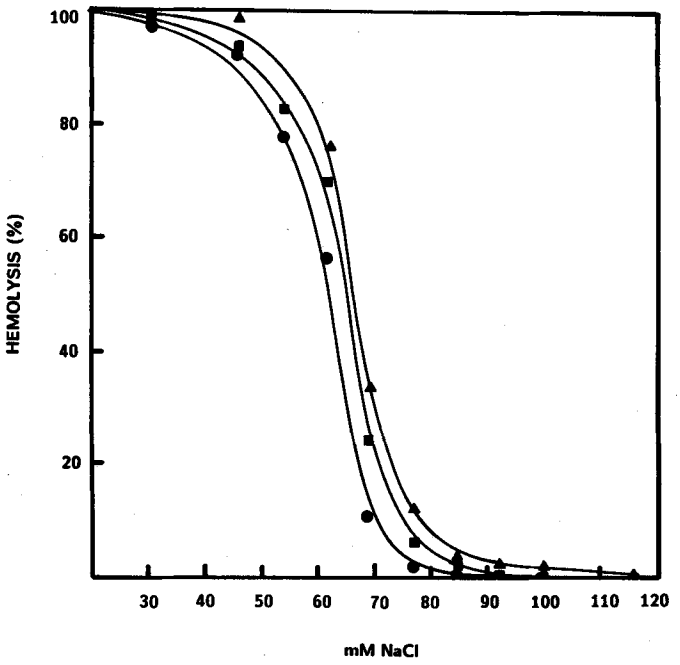

FIG. 1. Osmotic fragility curves of chicken erythrocytes: (●), control; (■), estrogen-treated; (▲), estrogen-treated and vitamin E-supplemented.

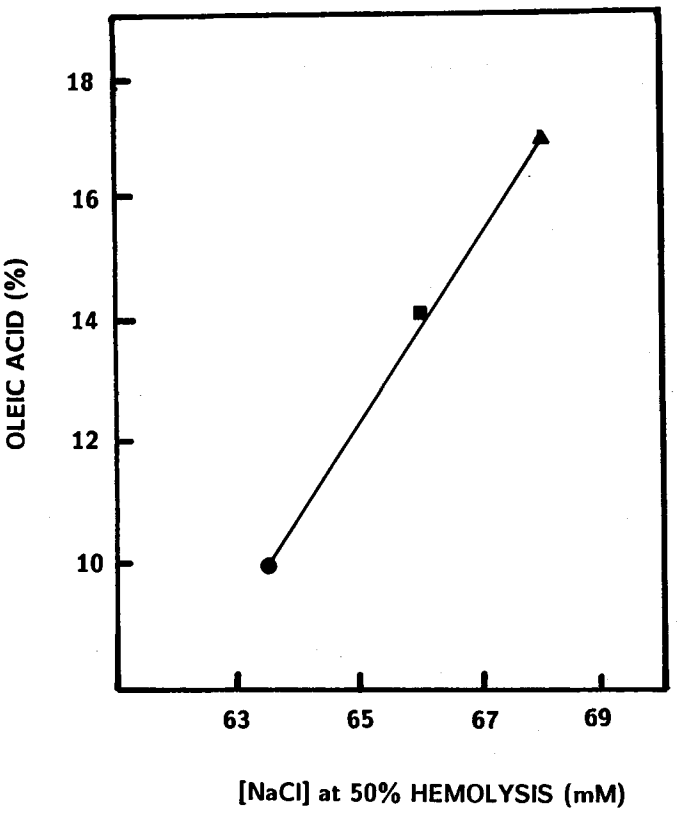

FIG. 2. Relationship between oleic acid content and osmotic fragility of erythrocytes: (●), control; (■), estrogen-treated; (▲), estrogen-treated and vitamin E-supplemented.

TABLE 3

Fatty Acid Composition of Erythrocyte Membrane Lipid in Chickens Implanted with Estrogen for Three Weeks (%)*

Fatty acid	Control	Estrogen	Estrogen + vit E
16:0	18.5 ± 0.6[a]	20.7 ± 0.4[b]	21.7 ± 0.9[b]
16:1ω7	1.0 ± 0.2	1.1 ± 0.3	1.0 ± 0.3
18:0	18.1 ± 0.4[a]	17.1 ± 0.6[b]	15.2 ± 1.0[c]
18:1ω9	10.0 ± 0.9[a]	14.2 ± 0.6[b]	16.9 ± 0.7[c]
18:1ω7	1.4 ± 0.1	1.3 ± 0.1	1.5 ± 0.1
18:2ω6	31.3 ± 0.9[a]	26.0 ± 1.3[b]	24.2 ± 0.5[b]
18:3ω3	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
18:4ω3	0.2 ± 0.1	—	0.2 ± 0.1
20:1ω9	0.4 ± 0.1	0.1 ± 0.1	0.3 ± 0.2
20:2ω6	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.1
20:3ω6	1.1 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
20:3ω3	1.2 ± 0.1	0.7 ± 0.1	1.2 ± 0.3
20:4ω6	9.8 ± 0.5	10.4 ± 0.5	9.4 ± 0.5
20:5ω3	0.1 ± 0.1	—	—
22:4ω6	2.3 ± 0.3	2.2 ± 0.4	2.6 ± 0.5
22:5ω6	1.1 ± 0.3	1.7 ± 0.7	1.5 ± 0.3
22:5ω3	0.7 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
22:6ω3	2.2 ± 0.2	3.0 ± 0.9	3.3 ± 0.2

*Mean ± standard deviation for five birds. Values on the same line bearing different superscripts are significantly different ($p < 0.05$).

Therefore, the presence of an increased level of peroxidation found in hyperlipidemia (8) may have a detrimental effect on membrane stability and aging of erythrocytes. Vitamin E deficiency also increases the rate of erythrocyte aging (28), and it seems reasonable that under certain conditions additional vitamin E might reduce the aging rate. As expected, there was an increase in erythrocyte fragility in the estrogen-implanted group, as there was an increase in plasma lipid peroxidation (Table 2). However, the addition of vitamin E to the diet, while it brought the plasma peroxidation levels down to normal, failed to reduce the erythrocyte fragility (Fig. 2). Studies by Mino et al. (30) on hyperlipidemic rats have shown that vitamin E is partitioned away from the erythrocytes and into the hyperlipidemic plasma. It is possible that, while the dietary level of supplemented vitamin E was sufficient to reduce the plasma lipid peroxidation, the partitioning effect seen by Mino et al. may have prevented the same degree of protection being given the erythrocytes.

Our present findings of increased plasma peroxidation and the occurrence of fatty acid compositional alterations in erythrocyte membranes under hyperlipidemic condition, accompanied by greater osmotic fragility, are of clinical significance, because an increased osmotic fragility of erythrocytes is noted in several blood diseases including hemolytic anemia (31). The association between hyperlipidemia and coronary heart disease has received great attention. However, little is known about the possible effects of hyperlipidemia on membrane function and aging of erythrocytes, which are questions that deserve further investigation.

ACKNOWLEDGMENT

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REFERENCES

1. Benga, G., and Holmes, R.P. (1984) *Prog. Biophys. Molec. Biol. 4,* 195–257.
2. Needland, P.J., and Clandinin, M.T. (1983) *Biochem. J. 212,* 573–583.
3. Holmes, R.P., Mahfouz, M.M., Travis, B.D., Yoss, N.L., and Keenan, M.J. (1983) *Ann. N.Y. Acad. Sci. 414,* 44–56.
4. Benga, G., Travis, B.D., Pop, V.I., Popescu, O., Toader, O., and Holmes, R.P. (1984) *Biochim. Biophys. Acta 775,* 255–259.
5. Hochstein, P., and Rice-Evans, C. (1982) in *Lipid Peroxides in Biology and Medicine* (Yagi, K., ed.) pp. 81–88, Academic Press, New York, NY.
6. Hochstein, P., and Jain, S.K. (1981) *Fed. Proc. 40,* 183–188.
7. Pfeffer, S.R., and Swislocki, N.I. (1982) *Mech. Ageing Dev. 18,* 355–367.
8. Smith, T.L., Toda, T., and Kummerow, F.A. (1985) *Atherosclerosis 57,* 119–122.
9. Cho, B.H.S. (1981) *Comp. Biochem. Physiol. 68B,* 19–23.
10. Tokuyasu, K., Imai, H., Taura, S., Cho, B.H.S., and Kummerow, F.A. (1980) *Arch. Pathol. Lab. Med. 104,* 41–45.
11. Vanstone, W.E., Dale, D.G., Oliver, W.F., and Commom, R.H. (1975) *Can. J. Biochem. 35,* 659–665.
12. Smith, E.R., Damassa, D.A., and Davidson, J.M. (1977) *Methods Psychobiol. 3,* 359–379.
13. Allain, C.C., Poon, L., Chan, S.H., Richmond, W., and Fu, P. (1974) *Clin. Chem. 20,* 470–475.
14. Foster, L.B., and Dunn, R.T. (1973) *Clin. Chem. 19,* 338–340.
15. Eng, L.F., and Noble, E.P. (1968) *Lipids 3,* 157–162.
16. Yagi, K. (1982) in *Lipid Peroxides in Biology and Medicine* (Yagi, K., ed.) pp. 232–242, Academic Press, New York, NY.
17. Hanahan, D.J., and Ekholm, J.E. (1974) *Methods Enzymol. 31,* 168–172.
18. Folch, J., Lees, M., and Sloane-Stanly, G.H. (1957) *J. Biol. Chem. 226,* 497–508.
19. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600–608.
20. Dacie, J.V., and Lewis, S.M. (1975) in *Practical Haematology,* 5th ed., pp. 220–208, Grune and Shatton, Inc., New York, NY.
21. Steel, R.G.D., and Torrie, J.H. (1960) in *Principles and Procedures of Statistics,* pp. 107–109, McGraw-Hill Book Company, Inc., New York, NY.
22. Reeves, R.D., Morris, M.D., and Barbour, G.L. (1976) *J. Am. Med. Assoc. 236,* 469–472.
23. Kudzma, D.J., Hegstad, P.M., and Stoll, R.E. (1973) *Metabolism 22,* 423–434.
24. Park, J.R., and Cho, B.H.S. (1988) *Lipids 23,* 327–333.
25. Waku, K., and Lands, W.E.M. (1968) *J. Lipid Res. 9,* 12–18.
26. Phillipis, G.B., Dodge, J.T., and Howe, C. (1969) *Lipids 4,* 544–549.
27. Ehrstrom, M., Harms-Ringdahl, M., and Alling, C. (1981) *Biochim. Biophys. Acta 644,* 175–182.
28. Chin, D., Lubin, B., and Shohet, S.B. (1982) *Free Rad. Biol. 5,* 115–160.
29. Pfeffer, S.R., and Swislocki, N.I. (1982) *Mech. Ageing Dev. 18,* 355–367.
30. Mino, M., Kasugai, O., and Shimizu, T. (1985) *Lipids 20,* 488–491.
31. Mentzer, W.C., Jr., and Clark, M.R. (1983) in *Biomembranes, Vol. 11, Pathological Membranes* (Nowotny, A., ed.) pp. 79–117, Plenum Press, New York, NY.

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Biosynthesis of Membrane Cholesterol During Peripheral Nerve Development, Degeneration and Regeneration[1]

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Biosynthesis of peripheral nerve cholesterol was investigated by the in vivo and in vitro incorporation of [1-^{14}C]-acetate into sciatic endoneurium of normal rats during development, degeneration and regeneration. Labeled sterols were rapidly formed (<10 min) within the endoneurial portion of sciatic nerve after [1-^{14}C]acetate administration by intraneural injection. The majority of labeled sterols were initially found in lanosterol and desmosterol. After six hr, the ^{14}C-labeling in both precursors was decreased to minimum, whereas cholesterol became the major labeled product of sterol. As myelination proceeded, the incorporation of [1-^{14}C]acetate into endoneurial cholesterol decreased rapidly and reached a minimum after six mo. In mature adult nerve, an increased proportion of biosynthesis of lanosterol and desmosterol also was demonstrated. The in vitro incorporation of [1-^{14}C]acetate into cholesterol was inhibited during Wallerian degeneration. Instead, cholesteryl esters were labeled as the major sterol product. Such inhibition, however, was not observed in the adult Trembler nerve (*Brain Res. 325,* 21–27, 1985), which is presumed to be due to a primary metabolic disorder of Schwann cells. The cholesterol biosynthesis was gradually resumed in degenerated nerve by either regeneration of crush-injured nerve or reattachment of the transected nerve. These results suggest that cholesterol biosynthesis in peripheral nerve relies on the axon to provide necessary substrates. De novo synthesis appears to be one of the major sources of endoneurial cholesterol that forms and maintains peripheral nerve myelin.
Lipids 23, 857–862 (1988).

Cholesterol is a major structural component of mammalian cell membranes. However, its functional role in neural tissue still is not completely understood. It may be important in the regulation and maintenance of various properties such as membrane fluidity and rigidity, numerous membrane bond enzymatic activities and cellular proliferation (1,2). Volpe and Obert (3) have suggested that a critical level of membrane cholesterol is essential for the expression of oligodendroglial differentiation and, perhaps, myelination. In peripheral nerve, the rate of lipogenesis does not change to the same extent for each lipid subclass during development, degeneration and regeneration. Cholesterol biosynthesis appears to be the most sensitive to aging (4) and to nerve fiber degeneration (4,5). It has been shown that substantial amounts of [1,2-^{3}H]cholesterol, incorporated into normal nerve during myelination, do not leave the nerve during Wallerian degeneration, but are retained and reutilized during nerve regeneration (6,7). An inhibition of cholesterol biosynthesis by *trans*-1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride (AY-9944) leads to a decrease in peripheral nerve myelination (8). Therefore, cholesterol plays a vital role in the myelinogenesis of peripheral nerve.

Spady and Dietschy (9) have demonstrated that liver is quantitatively far less important as a site for sterol synthesis than previously indicated and most sterol utilized by extra hepatic tissues is largely synthesized locally within those tissues. Considerable interests have been focused on the cholesterol biosynthesis and its regulation in various extra hepatic tissues (10–12). However, when $^{3}H_2O$ was used as a precursor, no incorporation was found in either brain (10) or sciatic nerve (4). The superiority of $^{3}H_2O$ over [^{14}C]acetate in studying cholesterol synthesis is, thus, limited to the nonneural tissues. Previously, we (4) have demonstrated that incorporation of [1-^{14}C]acetate without stimulation by any specific cofactors, provides a sensitive, convenient and reproducible method for studying in vitro lipid biosynthesis in peripheral nerve. Alternatively, endoneurial lipids in peripheral nerve can be labeled exclusively by endoneurial microinjection of radiolabeled precursor (13). The present studies were undertaken, utilizing both in vivo and in vitro incorporation of [1-^{14}C]acetate, to elucidate the metabolic significance of cholesterol biosynthesis during nerve development, degeneration and regeneration.

MATERIALS AND METHODS

Materials. [1-^{14}C]Acetate (sodium salt, 54–60 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). Lipid standards were purchased from NuChek (Elysian, MN) and Supelco (Bellefonte, PA). Silica Gel G_1 uniplates (250 μm) were purchased from Analtech (Newark, DE), and high-performance thin-layer plates (4.5 μm silica gel) with preadsorbent area were purchased from Whatman (Clifton, NJ). All solvents were high purity (HPLC grade) from Burdick and Jackson (Muskegon, MI).

In vivo incorporation of [1-^{14}C]acetate. The procedure used for endoneurial injection of [1-^{14}C]acetate was essentially the same as described by Yao (13). A nanoliter pump, consisting of controller, drive unit and pump chamber (model 1400 EC; W-P Instruments, Inc., New Haven, CT) was used for endoneurial microinjection. A glass capillary (1.0 ± 0.1 mm o.d., 4-inch length) was pulled, using a vertical pipette puller (model 700C; Kopf Instruments, Tunjunga, CA), to produce a needle tip of

[1]A preliminary report of this work was presented at the 17th Annual Meeting of the American Society for Neurochemistry held in Montreal, Quebec, Canada, on March 20, 1985. Part of this work was conducted while the author was associated with Mayo Clinic.

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Abbreviations: C, cholesterol; Ca, ceramide; CE, cholesteryl ester; Cs, coprostanol; Ds, desmosterol; 1,2-DG, 1,2-diacylglycerol; 1,3-DG, 1,3-diacylglycerol; FAME, fatty acid methyl ester; FFA, free fatty acid; GL, glycolipids; Ls, lanosterol; MG, monoacylglycerol; PL, phospholipids; TG, triacylglycerol; HPTLC, high-performance thin-layer chromatography; O, origin; S, std, standards; SF, solvent front; TLC, thin-layer chromatography.

5–15 μm o.d.. The barrel of this micropipette was placed inside the pump chamber, and both were filled with mineral oil just before the pipette tip was filled with delivery fluid. Care was taken to exclude air bubbles. [1-^{14}C]-Acetate was diluted in Evans blue solution (0.5 mg/ml) to yield a concentration of 1.1×10^7 dpm/μl. Under the dissecting microscope, the micropipette tip was placed inside the sciatic endoneurium (only one side was used for injection), after which 0.4 μl of [1-^{14}C]acetate solution was injected at a rate of 0.1 μl/min.

Tissue preparation. Unless otherwise indicated, one-mo-old male Sprague-Dawley rats were used throughout this study. Approximately 1 cm of sciatic nerve segment, including the injection site (blue dye served as marker), was removed at various times after injection. Each group consisted of four or more nerve samples. Immediately after removal, the sciatic nerve was desheathed on a cold plate (surface temperature 10 C) under a dissecting microscope. The desheathed portion (endoneurial tissue or a myelin-enriched portion) was blotted dry with filter paper before the wet wt was taken. The endoneurium was then lyophilized overnight to obtain the dry wt before lipid extraction.

Surgical procedure. Wallerian degeneration and regeneration were produced in rat sciatic nerve by either crush injury or transection, according to the methods described previously (14). Reattachment of transected nerve was according to the procedure of nerve xenograft described by Dyck et al. (15).

In vitro incorporation of [1-^{14}C]acetate. The procedure used for the in vitro incubation of sciatic endoneurium with [1-^{14}C]acetate was essentially the same as that described by Yao and Cannon (4). Immediately after removal, the proximal 3 cm of the sciatic nerve was desheathed on a cold plate under a dissecting microscope. The desheathed portions (endoneurial tissue) were placed in 1 ml of modified Kreb's glucose medium (16), pH 7.4. They were preincubated in a Dubnoff metabolic shaking incubator (ca. 50 strokes/min) at 37 C under a mixture of 95% O_2 and 5% CO_2. After 30 min, 200 μl of modified Kreb's glucose solution containing 2.5 μCi of [1-^{14}C]-acetate was added to the medium, and the incubation was continued for another 2 hr under the same conditions. At the end of this time, the nerve segments were removed and washed four times with ice-cold unlabeled medium before the procedure of lipid extraction was started.

Lipid extraction and separation. The lipids were extracted from sciatic endoneurium according to the method described previously (4,14). An aliquot was counted by a Beckman liquid scintillation counting system to obtain the total incorporation of labeled precursor into endoneurial lipids. Lipid profiles of sciatic endoneurium were analyzed by high-performance thin-layer chromatography (HPTLC), using method I of nonplar lipid separation described by Yao and Rastetter (17). The plate was first developed in solvent system I, consisting of benzene/diethyl ether/ethanol/acetic acid (60:40:1:0.05, v/v/v/v). The solvent front (SF) was allowed to migrate to 5.5 cm above the origin (O). After complete removal of acetic acid by hot air, the plate was developed in solvent system II, consisting of hexane/diethyl ether (94:6, v/v) to a groove scraped 7 cm above the preadsorbent layer (full length). After it was dried, the plate was finally developed to full length again in hexane alone. Sterols were identified by their comigration with standards on HPTLC using the solvent systems described above. Isolation of nonpolar lipids from total lipid extracts was achieved by column separations on Sep-Pak silica cartridge (17). Fatty acid methyl esters (FAME) were prepared by transesterification of lipid extracts, according to the method of Morrison and Smith (18). Distribution of radioactivity among lipid subclasses and methyl esters was determined by scraping lipid zones from thin-layer chromatography (TLC) plates into counting vials containing 1 ml of distilled water, to which was added 10 ml of Ready-Solv MP (Beckman, Irvine, CA) scintillation fluid. The amount of [1-^{14}C]acetate incorporated into nerve lipids was calculated using the specific activities of [1-^{14}C]acetate present in the incubation medium and was expressed as either the pmol of acetate or the total ^{14}C radioactivity (dpm) incorporated into nerve lipids per mg of wet tissue per hr.

Identification and distribution of radiolabeled lipids by fluorography. After HPTLC separation, the plate was sprayed with EN^{3}HANCE three times and covered with Glad Wrap (Union Carbide Corp., Danbury, CT). The plate then was exposed at −70 C for various times to Kodak X-Omat S or AR X-ray film in a Kodak X-Omatic cassettes with Du Pont Cronex Lightening-Plus intensifying screens (13,17). Quantification of fluorograms was carried out by LKB Ultrascan Laser Densitometer and Apple IIe computer.

RESULTS

In vivo cholesterol biosynthesis was investigated in rat sciatic nerve at various intervals after intraneurial microinjection of [1-^{14}C]acetate. Following removal of phospholipids (PL) and glycolipids (GL) by Sep-Pak silica cartridges, the distribution of ^{14}C-labeled nonpolar lipids in rat sciatic endoneurium was revealed by fluorography of high-performance thin-layer chromatograms (Fig. 1). Endoneurial triacylglycerol (TG), 1,3-diacylglycerol (1,3-DG), 1,2-diacylglycerol (1,2-DG), free fatty acid (FFA), ceramide (Ca) and sterols were rapidly labeled from

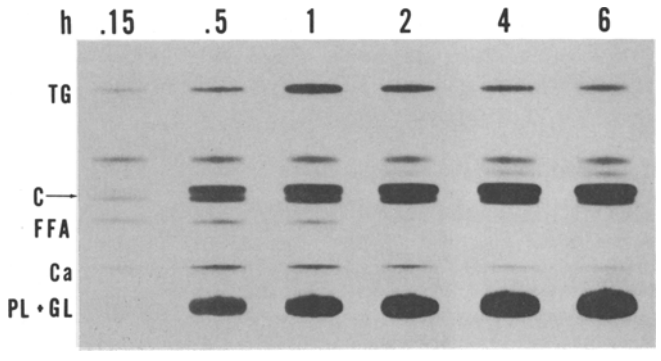

FIG. 1. Fluorograms of HPTLC of total ^{14}C-labeled lipids following microinjection of [1-^{14}C]acetate into rat sciatic endoneurium for various times (hr). Each sample represents total ^{14}C-labeled lipids extracted from 0.05 mg dry wt of endoneurium, except 0.15 hr (sample was extracted from 0.15 mg). After TLC development, the plate was exposed to Kodak X-Omat S film at −70 C for 4 days. Abbreviations: TG, triacylglycerol; C, cholesterol; FFA, free fatty acid; Ca, ceramide; PL, phospholipids; and GL, glycolipids.

[1-^{14}C]acetate following endoneurial injection. Most of labeled nonpolar lipids, except sterols, were all metabolized after 12 hr. Approximately one-fourth of total ^{14}C-labeled lipids were found to be endoneurial cholesterol.

To avoid possible contamination of radioactivity between sterols and acylglycerols, all acylated lipids were converted into FAME by transesterification before thin-layer separation. Figure 2 indicates that, in addition to cholesterol, lanosterol (Ls) and desmosterol (Ds) also were labeled from [1-^{14}C]acetate. A similar result also was obtained under in vitro conditions (data not shown). Those labeling in precursors of cholesterol were decreased gradually with time. Twelve hr after injection, over 80% of labeled sterols from [^{14}C]acetate were found to be cholesterol. The results concerning the incorporation of [1-^{14}C]acetate into fatty acids and PL have been published elsewhere (13).

To localize the site of cholesterol biosynthesis, [1-^{14}C]-acetate was incorporated in vitro into endoneurial tissue, as well as epi- and perineurial tissues. Figure 3 demonstrates that cholesterol biosynthesis in rat sciatic nerve was found mainly in myelin-enriched endoneurium. In addition to cholesterol, Ls and Ds, one of the cholesterol metabolites—coprostanol (Cs)—also was labeled in endoneurium. This was not revealed in previous fluorograms (Fig. 2), due to a shorter exposure time to X-ray film. On the other hand, the majority of ^{14}C-labels in the epi- and perineurial lipids were acylglycerols, i.e., TG, 60% and 1,2-DG, 30%.

Peripheral nerve cholesterol biosynthesis decreased rapidly with increasing age (Fig. 4). After one month, the decrease continued, but at a much lower rate. The in vitro rate of [^{14}C]acetate into endoneurial sterols was >13 times higher in 10-day-old rats ($9.91 \pm 0.48 \times 10^{-4}$ dpm/mg wet wt/hr) than in four-mo-old rats ($0.73 \pm 0.11 \times 10^{-4}$ dpm/mg wet wt/hr). In mature adult nerve, there was an increased proportion of ^{14}C-label in Ls and Ds (Fig. 5).

To test whether the inhibited cholesterol biosynthesis in injured nerve can be induced by surgical correction, the in vitro incorporation of [1-^{14}C]acetate into endoneurial cholesterol was investigated in rat sciatic nerve with and without reattachment after 14 days transection (Table 1). Rat sciatic nerves were transected for 14 days to ensure that cholesterol biosynthesis was minimized before surgical reattachment. In control experiments, the transected nerves were allowed to degenerate for another 14 days and 30 days. The total incorporation of [1-^{14}C]acetate into endoneurial lipids was substantially lower than that of contralateral intact nerves. The majority of labeled cholesterols were in the form of cholesteryl esters. (CE).

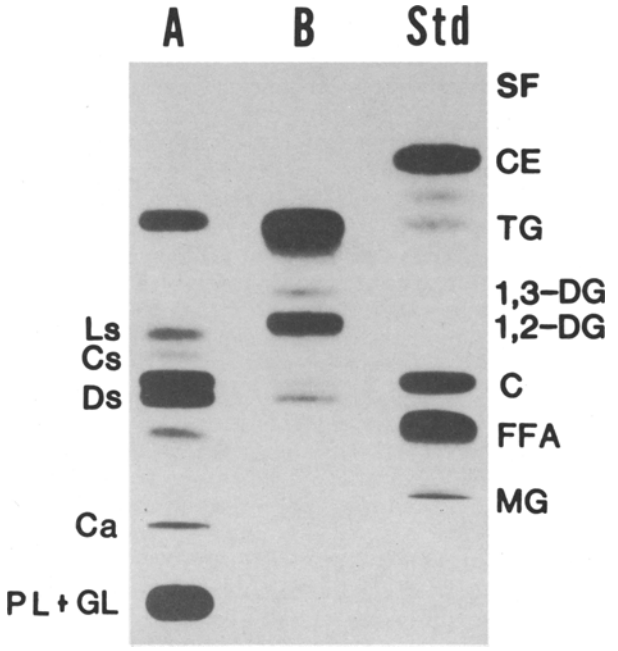

FIG. 3. Fluorograms of HPTLC of total ^{14}C-labeled lipids following 2 hr in vitro incorporation of [1-^{14}C]acetate into endoneurium (A) and epineurium and perineurium (B) of rat sciatic nerve. Ca. 5,000 cpm/sample were applied to a thin-layer plate which was exposed to Kodak X-Omat S film at −70 C for 7 days. Abbreviations: Std, standards; SF, solvent front; CE, cholesteryl ester; TG, triacylglycerol; 1,3-DG, 1,3-diacylglycerol; 1,2-DG, 1,2-diacylglycerol; Ls, lanosterol; Cs, coprostanol; C, cholesterol; Ds, desmosterol; FFA, free fatty acids; MG, monoacylglycerol; Ca, ceramide; PL, phospholipids; and GL, glycolipids.

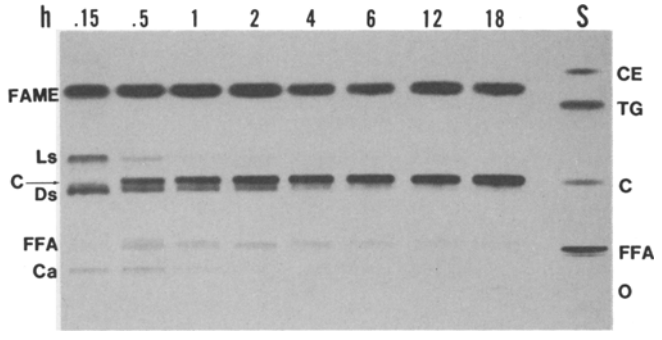

FIG. 2. Separation of ^{14}C-labeled sterols on HPTLC after methanolysis of total ^{14}C-labeled lipids at various times following endoneurial microinjection of [1-^{14}C]acetate. Ca. 7,000 cpm/sample were applied to a thin-layer plate which was exposed to Kodak X-Omat S film at −70 C for 2 days. Abbreviations: S, standards; CE, cholesteryl ester; FAME, fatty acid methyl ester; TG, triacylglycerol; Ls, lanosterol; C, cholesterol; Ds, desmosterol; FFA, free fatty acid; Ca, ceramide; and O, origin.

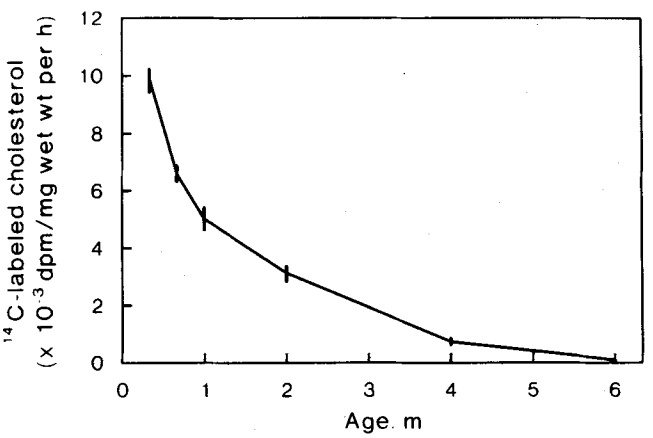

FIG. 4. Effect of age on cholesterol biosynthesis following 2 hr in vitro incorporation of [1-^{14}C]acetate into endoneurial lipids of rat sciatic nerve.

On the other hand, there was a marked increase of [1-^{14}C]acetate incorporation into total endoneurial lipids of transected nerve which was surgically reattached for 30 days. Concomitantly, the level of cholesterol biosynthesis was resumed, and the proportion of labeled CE also was substantially reduced. The induction of cholesterol biosynthesis was further confirmed by fluorography following HPTLC separation (Fig. 6A). In addition, marked decrease of endoneurial CE in reattached nerve also was revealed by charred HPTLC (Fig. 6B).

To test whether cholesterol biosynthesis is affected by Schwann cell disorder, in vitro [1-^{14}C]acetate incorporation into endoneurial cholesterol was compared between Trembler mutant and Wallerian degeneration models (Table 2). Incorporation of [1-^{14}C]acetate into endoneurial cholesterol reached its minimum level in adult rat sciatic nerve seven days after crush injury or 60 days after transection. By contrast, cholesterol biosynthesis in adult Trembler nerve was not significantly different than that of normal controls (19). On the other hand, the level of CE formation was elevated more significantly in both models than in normal controls.

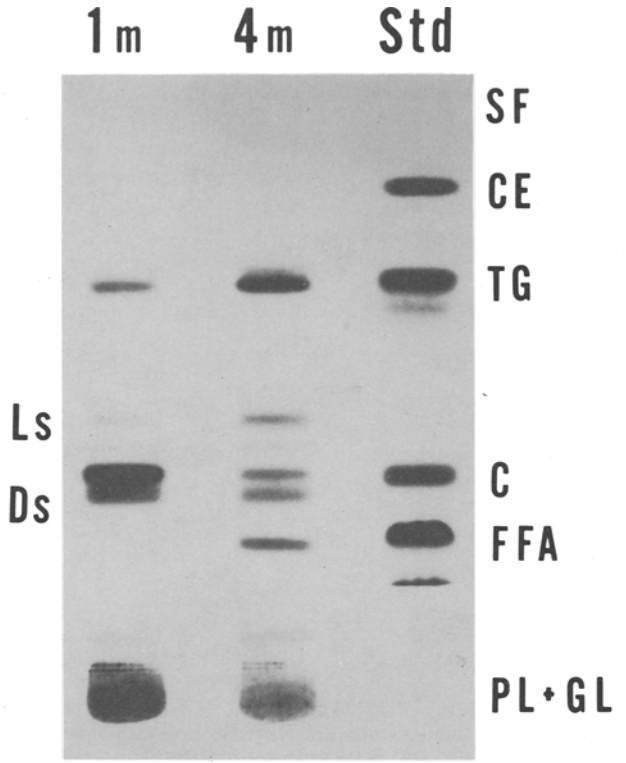

FIG. 5. Fluorograms of HPTLC of total ^{14}C-labeled lipids following 2 hr in vivo incorporation of [1-^{14}C]acetate into endoneurial lipids of young (1-month-old) and mature (4-month-old) adult rat sciatic nerves. Each sample represents total labeled lipid extracts from 0.25 mg wet wt of endoneurium. After TLC development, the plate was exposed to Kodak X-Omat S film at −70 C for 3 days. Abbreviations: SF, solvent front; CE, cholesteryl ester; TG, triacylglycerol; Ls, lanosterol; C, cholesterol; Ds, desmosterol; FFA, free fatty acid; PL, phospholipids; and GL, glycolipids.

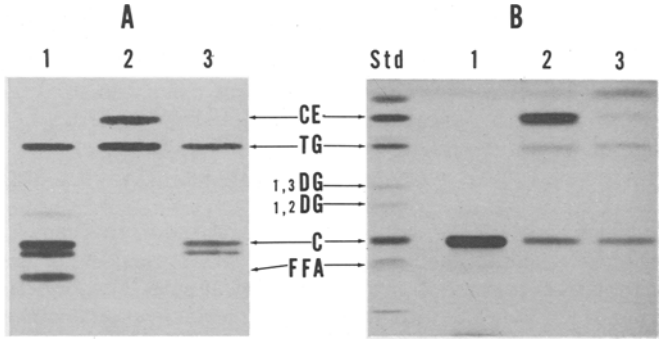

FIG. 6. HPTLC of nonpolar lipids separation by fluorography (A) and by charring (B) following 2 hr in vitro incorporation of [1-^{14}C]acetate into endoneurial lipids of control nerve-contralateral uninjured (1), injured nerve-30 days after transection (2), and regenerated nerve—30 days after reattachment of transected nerve (3). Ca. 5,000 cpm/sample were applied to thin layer plates. After development, one plate was sprayed with 10% (wt/vol) curpic sulfate in 8% (wt/vol) phosphoric acid and charred at 180 C for 10 min. The other plate was exposed to Kodak X-Omat S film at −70 C for 4 days. Abbreviations: CE, cholesteryl ester; TG, triacylglycerol; 1,3-DG, 1,3-diacylglycerol; 1,2-DG, 1,2-diacylglycerol; C, cholesterol; and FFA, free fatty acid.

TABLE 1

In Vitro Incorporation of [1-^{14}C]Acetate into Endoneurial Lipids of Transected and Reattached Rat Sciatic Nerves[a]

	14 days (n = 5)				30 days (n = 4)			
	Controls		Reattached		Controls		Reattached	
Labeled lipids	L[b]	R[b]	L	R	L	R	L	R
Total lipids, $\times 10^{-4}$ dpm/nerve	15.0 ± 2.4	n.a.[c]	13.7 ± 2.5	5.8 ± 1.2	8.0 ± 3.3	1.1 ± 0.2	10.8 ± 2.3	18.0 ± 8.1
Cholesterol								
Free, %	26.2 ± 1.7	1.7 ± 0.2	25.3 ± 2.4	5.4 ± 1.9	18.4 ± 1.8	4.3 ± 2.1	19.5 ± 1.0	20.2 ± 5.5
Esterified, %	0.4 ± 0.1	13.7 ± 1.7	0.5 ± 0.1	11.4 ± 1.8	0.3 ± 0.2	8.7 ± 1.3	0.3 ± 0.1	4.1 ± 2.1

[a]Following 12 days transection, distal nerves of control group were allowed to degenerate for another 14 days and 30 days, respectively. Nerves from reattached group were regenerated after surgical reattachment for 14 days and 30 days, respectively.

[b]L, contralateral uninjured nerves; R, transected nerves.

[c]Not available.

TABLE 2

In Vitro Cholesterol Biosynthesis in Sciatic Endoneurium Following [1-^{14}C]Acetate Incorporation

	Schwann cell defect		Axonal degeneration[a]			
			Acute injury		Permanent transection	
Lipid classes	Control[b]	50-day-old Trembler mouse[b]	Control	7 days after crush	Control	60 days after transection
Total ^{14}C-labeled lipids × 10^{-4} dpm/nerve/hr	1.9[c]	2.1	14.0	2.5	5.1	4.9
Cholesterol						
Free, %	14.9	13.5	29.0	2.4	23.7	3.1
Esterified, %	1.3	5.5	0.5	5.5	0.7	16.2

[a]From Sprague-Dawley rats.

[b]Values were calculated from previously published data (18).

[c]Mean value of 3-5 samples for all experiments.

DISCUSSION

Previously, our laboratory has shown that exclusive labeling of endoneurial lipids in peripheral nerve by endoneurial microinjection of radiolabeled precursor provides a unique model for studying metabolic turnover of fatty acids and acylglycerols in rat sciatic nerve (13). The present paper is an extension of our previous studies, demonstrating that peripheral nerve is capable of de novo biosynthesis of cholesterol. A temporal association between decreased ^{14}C-label in Ls and Ds, and increased ^{14}C-label in cholesterol (Fig. 2), suggests that the overall pathway of cholesterol biosynthesis in peripheral nerve appears to be the same as that in liver (20). This result is in accordance with the recent findings by Bourre et al. (21), demonstrating that both 7-dehydrodesmosterol and Ds are present in developing rat sciatic nerve and nearly absent in mature adult nerve.

Labeled glucose (22) and leucine (23,24) also have been utilized to synthesize cholesterol in peripheral nerve, because a common intermediate-acetyl CoA can be generated by both precursors. In brain, ketone bodies serve as important precursors of myelin cholesterol (25-27). It is not clear, however, whether a direct metabolic path from ketone bodies via acetoacetyl CoA toward formation of cholesterol exists in peripheral nerve. Squalene, which was not normally found in the [1-^{14}C]acetate metabolites, became a major product in the presence of excessive NADP$^+$ (28), which suggests that epoxidation of squalene may be a key step in the regulation of cholesterol biosynthesis in the peripheral nervous system.

The level of cholesterol biosynthesis is highest in the developing peripheral nerve and decreases rapidly as myelination proceeds (Fig. 4). Furthermore, there is an increased proportion of biosynthesis of cholesterol precursors (Fig. 5) with increasing age. This incomplete biosynthesis of endoneurial cholesterol may imply a decreased activity in certain specific enzymes, e.g., Ds reductase, in adult peripheral nerve. Although the mature adult nerve maintains a minimum activity of cholesterol biosynthesis, the ability to synthesize sterols continually even after myelination (Fig. 5) suggests that adult peripheral nerve myelin is not a stable metabolic entity. In the absence of myelin assembly, cholesterol biosynthesis is completely abolished. The inhibited biosynthesis, however, can be resumed by regeneration of injured nerve (Fig. 7) or by surgical reattachment of transected nerves (Fig. 6). On the other hand, the adult Trembler nerve, which is presumed to be due to a metabolic disorder of Schwann cells (29,30), do not exhibit a defect in cholesterol biosynthesis (19). These data indicate that cholesterol biosynthesis may be dependent on the axon as the major source of substrate and the level of membrane cholesterol is essential for expression of myelinogenesis.

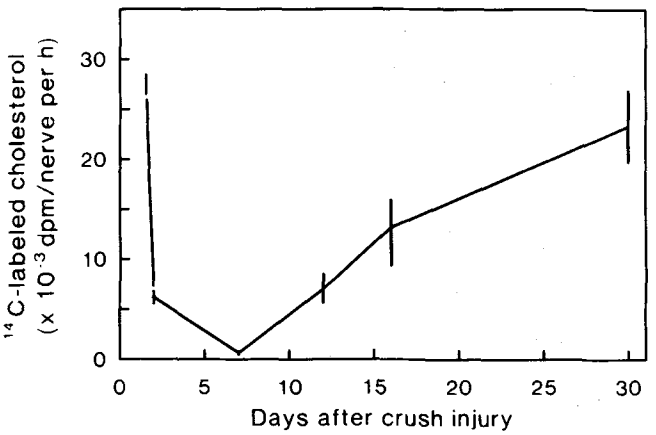

FIG. 7. In vitro cholesterol biosynthesis in rat sciatic endoneurium during Wallerian degeneration and regeneration. Data were calculated from previously published data (4).

One of the major biochemical events associated with demyelination is the temporal changes in membrane cholesterol with histological observations. In general, nerve fiber degeneration is accompanied by a decrease in free cholesterol and an increase in CE that return to normal levels on regeneration (14,31). The altered levels of endoneurial cholesterol are associated temporally with the rate of [1-^{14}C]acetate incorporation into endoneurial cholesterol (4). On the other hand, an increase in cholesterol esterifying activity, which is temporally associated with axonal degeneration (32), is primarily responsible for

cholesterol esterification in degenerated nerve. During remyelination, CE hydrolysis, which is active in normal nerve (33), appears to overshadow cholesterol esterification. Thus, it is possible that, to detoxify the increased FFA during demyelination (31), cholesterol is stored as CE and then is reutilized in remyelination after hydrolysis (14,34). In summary, de novo synthesis of cholesterol from acetate and hydrolysis of CE formed during degeneration appear to be the two major sources of endoneurial cholesterol that are used to form new myelin. Alternatively, cholesterol incorporated from blood stream by the Schwann cells (6,35), already present in nerve before degeneration (6,7) and transported via axoplasmic flow (36–38), also may be utilized to form new myelin.

REFERENCES

1. Chen, H.W., Kandutsch, A.A., and Heiniger, H.J. (1978) *Prog. Exp. Tumor Res. 22,* 275–316.
2. Coleman, P.S., and Lavietes, B.B. (1981) *CRC Crit. Rev. Biochem. 11,* 341–393.
3. Volpe, J.J., and Obert, K.A. (1983) *J. Neurochem. 40,* 530–537.
4. Yao, J.K., and Cannon, K.P. (1983) *Biochim. Biophys. Acta 753,* 331–338.
5. Rawlins, F.A., and Smith, M.E. (1971) *Neurobiology 1,* 225–231.
6. Rawlins, F.A., Hedley-Whyte, E.T., Villegas, G., and Uzman, B.G. (1970) *Lab. Invest. 22,* 237–240.
7. Rawlins, F.A., Villegas, G.M., Hedley-Whyte, E.T., and Uzman, B.G. (1972) *J. Cell. Biol. 52,* 615–625.
8. Rawlins, F.A., and Uzman, B.G. (1970) *Lab. Invest. 23,* 184–189.
9. Spady, D.K., and Dietschy, J.M. (1983) *J. Lipid Res. 24,* 303–315.
10. Anderson, J.M., and Dietschy, J.M. (1979) *J. Lipid Res. 20,* 740–752.
11. Turley, S.D., Andersen, J.M., and Dietschy, J.M. (1981) *J. Lipid Res. 22,* 551–569.
12. Heacock, A.M., Klinger, P.D., Seguin, E.B., and Agranoff, B.W. (1984) *J. Neurochem. 42,* 987–993.
13. Yao, J.K. (1985) *J. Neurochem. 45,* 589–595.
14. Yao, J.K., Natarajan, V., and Dyck, P.J. (1980) *J. Neurochem. 35,* 933–940.
15. Dyck, P.J., Lais, A.C., Sparks, M.F., Oviatt, K.F., Hexum, L.A., and Steinmuller, D. (1979) *Neurology 29,* 1215–1221.
16. Plesure, D.E., and Towfighi, J. (1972) *Arch. Neurol. 29,* 1215–1221.
17. Yao, J.K., and Rastetter, G.M. (1985) *Anal. Biochem. 150,* 111–116.
18. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600–608.
19. Yao, J., and Bourre, J.M. (1985) *Brain Res. 325,* 21–27.
20. Gaylor, J.L. (1972) *Adv. Lipid Res. 10,* 89–141.
21. Bourre, J.M., Clement, M., Gerard, D., and Chaudiere, J. (1988) *Trans. Am. Soc. Neurochem. 19,* 225.
22. Smith, M.E. (1969) *J. Neurochem. 16,* 83–92.
23. Rawlins, F.A., and Smith, M.E. (1971) *J. Neurochem. 18,* 1861–1870.
24. Stillway, L.W., Weigand, D.A., and Buse, M.G. (1979) *Lipids 14,* 127–131.
25. Edmond, J. (1974) *J. Biol. Chem. 249,* 72–80.
26. Yeh, Y-Y., Slreuli, V.L., and Zee, P. (1977) *Lipids 12,* 957–964.
27. Koper, J.W., Lopes-Cardozo, M., and van Golde, L.M.G. (1981) *Biochim. Biophys. Acta 666,* 411–417.
28. Yao, J.K. (1983) *J. Neurochem. 41* (Suppl.), 5154.
29. Low, P.A. (1976) *J. Neurol. Sci. 30,* 327–341.
30. Low, P.A. (1976) *J. Neurol. Sci. 30,* 343–368.
31. Yao, J.K. (1984) in *Peripheral Neuropathy* (Dyck, P.J., Thomas, P.K., Lambert, E.H., and Bunge, R., eds.), 2nd edn., pp. 510–530, W.B. Saunders Co., Philadelphia, PA.
32. Yao, J.K., and Dyck, P.J. (1981) *J. Neurochem. 37,* 156–163.
33. Mezei, C. (1970) *J. Neurochem. 17,* 1163–1170.
34. Bowen, D.M., Davison, A.N., and Ramsey, R.B. (1974) in *Biochemistry of Lipids* (Goodwin, T.W., ed.) Vol. 4, pp. 141–179, University Park Press, Baltimore, MD.
35. Svanberg, O. (1970) *Acta Physiol. Scand. 80,* 45–49.
36. McGregor, A., Jeffrey, P.L., Klingman, J.D., and Austin, L. (1973) *Brain Res. 63,* 466–469.
37. Wood, P.L., and Boegman, R.J. (1980) *FEBS Lett. 115,* 110–112.
38. Toews, A.D., Saunders, B.F., Blaker, W.D., and Morell, P. (1983) *J. Neurochem. 40,* 555–562.

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Dexamethasone-induced Alterations in the Glycosphingolipids of Rat Kidney

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To determine whether glucocorticoids would influence the glycosphingolipid composition of the rat kidney, male albino rats of the Sherman strain were subcutaneously administered dexamethasone (100 μg/100 g body wt/day) or diluent for four days. The compositions of ceramide and of acidic and neutral glycosphingolipids of the kidneys of these animals were then examined and compared. The results demonstrated that dexamethasone administration: 1) increased the content of ceramide and of acidic and neutral glycosphingolipids in kidney; 2) increased the relative percentage of globotriaosyl- and globotetraosylceramide, but decreased the relative percentages of glucosylceramide; 3) decreased the relative percentages of G_{M3} and increased other gangliosides; 4) increased the relative percentages of *N*-glycolylneuraminic acid in G_{M3}; 5) did not appear to influence significantly the long-chain bases of the major glycosphingolipids; and 6) altered the relative percentages and chain length of the hydroxy and nonhydroxy fatty acids of the major acidic and neutral glycosphingolipids in this tissue. The data show that dexamethasone administration induces quantitative and qualitative changes in the glycosphingolipids of the rat kidney.
Lipids 23, 863-868 (1988).

Previously, the mammalian kidney has been shown to be rich in glycosphingolipids (1-5). While their functional significance remains to be elucidated, prior studies from a number of laboratories (6-9) have suggested that these lipids may play a role in many important cellular processes in various tissues, including the kidney. For example, Spiegel et al. (10) have recently implicated glycosphingolipids as modulators of sodium channels in the apical membranes of A_6 cells, a cultured kidney epithelial cell line. In the rat kidney, earlier studies (1) have suggested that alterations in the glycosphingolipids may be associated with the aging process.

Relatively few studies have been undertaken with the aim to modulate the glycosphingolipid composition of mammalian kidney. In this regard, sex hormones have been shown to influence the glycosphingolipids of the rat and mouse kidney (1-5). Recent studies by our laboratory (11) and others (12) also have shown that glucocorticoid hormones may influence the glycosphingolipid composition of various tissues, including the rat small intestine (11). To date, however, data concerning the influence of these latter hormones on the glycolipids of the rat kidney are lacking. The present experiments were undertaken to characterize the effects of the synthetic glucocorticoid, dexamethasone, on the acidic and neutral glycosphingolipid composition of the rat kidney. The results of these investigations, a comparison of the effects of this hormone on the glycolipids of rat kidney with those previously noted in rat small intestine (11), and a discussion of their potential physiological significance are presented.

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Abbreviations: GLC, gas-liquid chromatograph(y); HPTLC, high-performance thin-layer chromatography; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. Dexamethasone was purchased from Sigma Chemical Co. (St. Louis, MO). Sphingosine standards, 3% OV-1 on Anakron ABS and ceramide were purchased from Analabs (North Haven, CT). Phytosphingosine was obtained from Calbiochem (La Jolla, CA). Florisil (60-100 mesh) and fluorescamine (Fluram-Roche) were obtained from Fisher Scientific (Fairlawn, NJ). Mixtures of nonhydroxy and hydroxy fatty acid standards and EGSS-X 10% on Gas Chrom P and Power-Sil-Prep were obtained from Alltech/Applied Science Lab. Inc. (State College, PA). The ganglioside standards G_{M1}, G_{D1a}, G_{T1a}, and G_{T1b} were purchased from Supelco (Bellefonte, PA). G_{M2}, isolated from Tay-Sachs brain, was a gift from Dr. G. Dawson. G_{M3} was isolated from rat small intestine and purified by the method of Glickman and Bouhours (13). Glucosyl-, lactosyl-, globotriaosyl-, and globotetraosylceramide, purified from human erythrocytes, were gifts from Dr. S. K. Kundu (14). All other chemicals were purchased from Fisher Scientific or Sigma Chemical Co., unless otherwise indicated.

Lipid extraction. Two groups of 16 male albino rats of the Sherman strain, weighing 200-250 g, were injected subcutaneously with vehicle (0.9% saline) or dexamethasone (100 μg/day100 g body wt) for four days. The last injection was administered two hr before the animals were killed (11). With water ad libitum, the animals were fasted overnight, killed rapidly by cervical dislocation and their kidneys were excised. The kidneys were decapsulated and papillae removed, then sliced and washed with saline to remove blood clots. The glycosphingolipids of the kidneys of each group were extracted as previously described (11,15). Briefly, the samples were homogenized with methanol in a Waring Blender, and the glycolipids extracted overnight with 20 vol of chloroform/methanol (2:1, v/v) (15). The protein residue was collected by filtration and reextracted with chloroform/methanol (1:2, v/v). The filtrates were combined, dried in a rotary evaporator and redissolved in chloroform/methanol (2:1, v/v) for further analysis.

Glycosphingolipid, ceramide purification and identification by high-performance thin-layer chromatography (HPTLC). Total lipid extracts were partitioned with water and the lower phase washed five times with Folch upper phase (chloroform/methanol/water, 3:48:47, v/v/v) to insure complete extraction of gangliosides into the upper phase (15). The upper-phase gangliosides were then purified by passage through Sep-pak cartridges as described previously (16).

The neutral glycolipids and ceramide were purified by chromatography of the acetylated neutral lipids on a Florisil column (17). The gangliosides, ceramide and the deacetylated neutral lipids were analyzed by HPTLC on Silica Gel 60 precoated plates (E. Merck, Darmstadt, West Germany) in the solvent system chloroform/methanol/water with 1% $CaCl_2$ (20:35:7, v/v/v) (18).

By brief exposure to iodine vapor, individual neutral glycolipids and ceramide were identified and then quantified, after scraping the silica gel area corresponding to their position following migration. The number of sphingoid bases liberated by methanolysis was determined by fluorometry, following reaction of the free bases with fluorescamine (17). Quantification of the various gangliosides was performed using a Beckman densitometer as described by Mullin et al. (18). In the present experiments, all of the bands from each ganglioside species were analyzed. Using radiolabeled glycosphingolipids, recoveries were determined from tissue homogenates, as previously described by Suzuki et al. (14). Recoveries for G_{M3}, ceramide, glucosyl-, lactosyl-, globotriaosyl- and globotetraosylceramide were 77 ± 4, 82 ± 3, 82 ± 5, 80 ± 3, 83 ± 6 and 81 ± 5%, respectively (N = 4). The variations in recoveries of these various lipids were not found to be significantly different.

Desialylation of G_{M3}. Desialylation of purified G_{M3} was performed by acid hydrolysis using 0.1 M H_2SO_4 at 80 C for 1.5 hr, as previously described (19). On HPTLC plates, N-glycolyl and N-acetylneuraminic acids were separated, using a solvent system of n-propyl-alcohol/water/ammonium hydroxide (60:28:1.5, v/v/v) (20), and visualized with resorcinol.

Analysis of carbohydrate moieties of G_{M3} and neutral glycolipids. Carbohydrate moieties were identified by gas-liquid chromatographic analysis of their trimethylsilyl derivatives, as previously described (13,21).

Analysis of fatty acids and long-chain bases of glycosphingolipids and ceramide. Individual glycosphingolipids and ceramide were separated and identified by HPTLC and, after scraping the silica gel areas corresponding to their positions, were hydrolyzed in 1 ml of dry methanolic HCl (0.75 N) at 80 C for 16 hr. This was followed by extraction of the fatty acid methyl esters by hexane (22). The methanolic phase then was alkalinized with aqueous NaOH and the long-chain bases were extracted by diethyl ether (23).

Nonhydroxy and hydroxy fatty acids were separated by chromatography on a Florisil column, as previously described (24). In certain experiments, a known amount of C_{21} fatty acid methyl ester was added as an internal standard to quantitate the nonhydroxy and hydroxy fatty acids in the samples (25). Nonhydroxy fatty acid methyl esters were dissolved in a small volume of hexane, and hydroxy fatty acid methyl esters were silylated prior to chromatography (25). Analyses of both nonhydroxy and hydroxy fatty acids were performed on a Hewlett-Packard 5790A gas-liquid chromatograph (GLC) equipped with a flame ionization detector and interfaced with a Hewlett-Packard 3390A integrator, as previously described (25). Peak identification was based on retention times, identical to known standards run under the same conditions (25).

Long-chain bases, free from fatty acids, were dissolved in chloroform, washed with 0.1 M NaOH and then washed repeatedly with water. The chloroform phase was dried under N_2 and the residue silylated, as described by Carter and Gaver (26). The long-chain bases then were analyzed by GLC on an OV-1 column operated with temperature programming from 245 to 305 C at 3 C per min (25). Identification was based on retention times, identical to known standards run under the same conditions (25).

Statistical methods. Unless otherwise indicated, all results were expressed as mean value ± SE. Paired or unpaired t-tests were used for all statistical analysis. $P < 0.05$ was considered significant.

RESULTS

Analyses of kidney glycosphingolipids and ceramide in control and dexamethasone-treated animals. In agreement with earlier studies of control animals (1), glucosyl- and globotetraosylceramide were found to be the principal neutral glycosphingolipids, and hematoside (G_{G3}) was the major ganglioside of this organ (Tables 1 and 2,

TABLE 1

Contents and Relative Percentages of Ceramide and Neutral Glycosphingolipids in Kidneys of Dexamethasone-treated and Control Rats[a]

	Control		Dexamethasone-treated	
	Content	%	Content	%
Ceramide	0.18 ± 0.03	18.6	0.25 ± 0.03	16.1
Glucosylceramide	0.23 ± 0.03	23.7	0.27 ± 0.02	17.4
Lactosylceramide	0.06 ± 0.01	6.2	0.08 ± 0.01	5.2
Globotriaosylceramide	0.10 ± 0.01	10.3	0.21 ± 0.04[b]	13.5
Globotetraosylceramide	0.40 ± 0.06	41.2	0.73 ± 0.06[b]	47.1
Total	0.97 ± 0.06		1.55 ± 0.05[b]	

[a]Content values are expressed in μg/mg protein and represent mean ± SE for four separate preparations (four rats/preparation) of each group. Quantitation and recoveries of ceramide and the individual glycosphingolipids were determined as previously described by Saito and Hakomori (17) and Suzuki et al. (14), respectively.

[b]$P < 0.05$ compared to control values.

TABLE 2

Contents and Relative Percentage of G_{M3} in Kidneys of Dexamethasone-treated and Control Rats[a]

	Control		Dexamethasone-treated	
	Content	%	Content	%
G_{M3}	1.64 ± 0.16	76.0	2.18 ± 0.13[b]	67.1
Others	0.51 ± 0.07	23.7	1.06 ± 0.15[b]	32.6
Total	2.16 ± 0.18		3.25 ± 0.22[b]	

[a]Values are expressed in nmol NeuAc/mg protein and represent mean ± SE of four separate preparations (four rats/preparation) of each group. Quantitation and recoveries of G_{M3} and the other gangliosides were determined as previously described by Mullin et al. (18) and Suzuki et al. (14), respectively.

[b]$P < 0.05$ compared to control values.

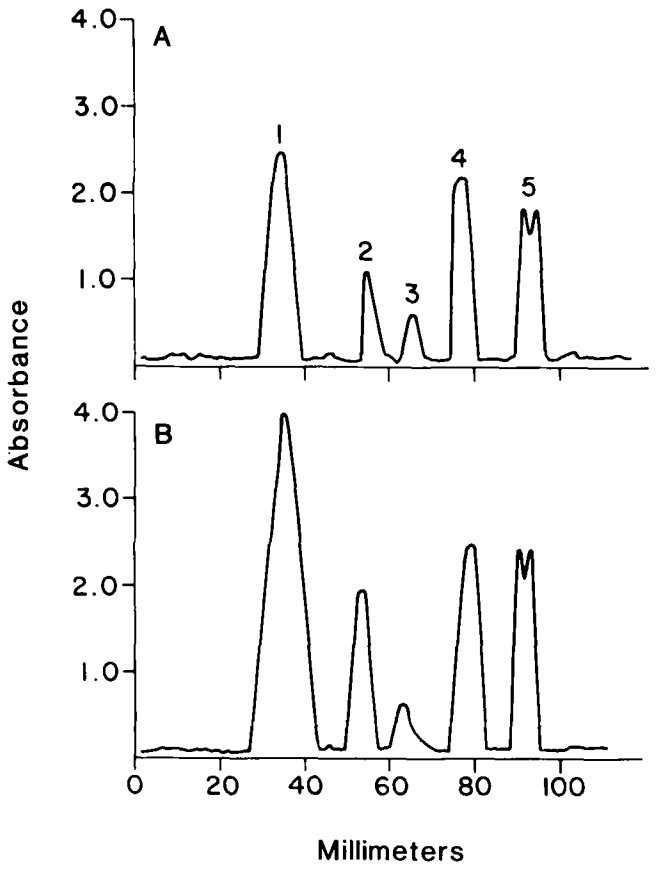

FIG. 1. Representative densitometric tracings of: A, control and dexamethasone-treated B, kidney ceramide and neutral glycosphingolipids. 1, Globotetraosylceramide; 2, globotriaosylceramide; 3, lactosylceramide; 4, glucosylceramide; and 5, ceramide.

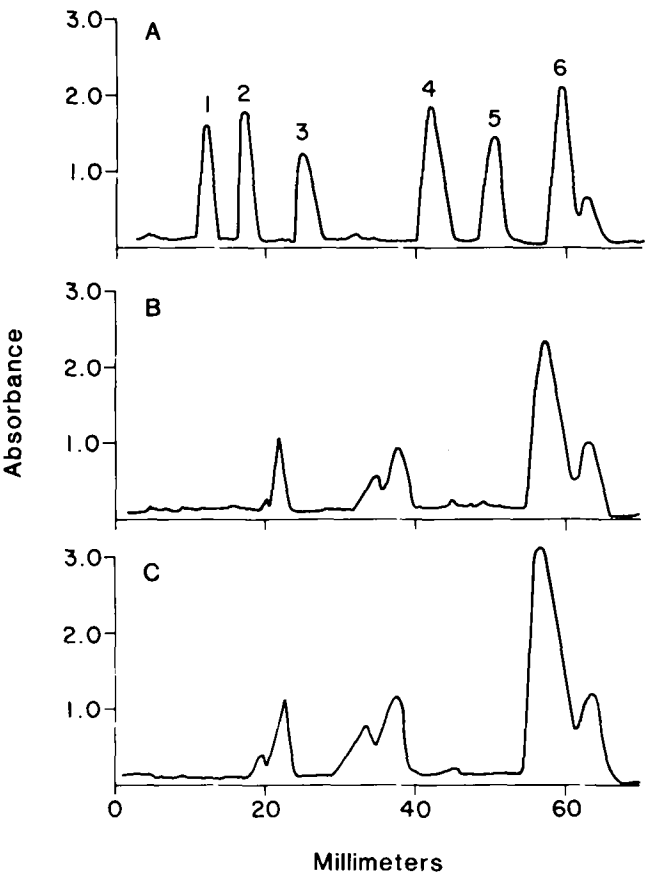

FIG. 2. Representative densitometric tracings of: A, standards; B, control; and C, dexamethasone-treated gangliosides. 1, GT_{1b}; 2, GT_{1a}; 3, GD_{1a}; 4, G_{M1}; 5, G_{M2}; and 6, G_{M3}.

Figs. 1 and 2). Smaller amounts of lactosyl- and globotriaosylceramide, as well as ceramide, a nonsugar containing compound, also were present (Table 1, Fig. 1). In dexamethasone-treated kidneys, the total content of neutral glycosphingolipids and ceramide was found to be significantly increased when compared with control kidneys (Table 1, Fig. 1). Moreover, the relative percentages of globotriaosyl- and globotetraosylceramide were increased by dexamethasone, and, after administration of this synthetic glucocorticoid for four days, the relative percentage of glucosylceramide was decreased.

As shown in Table 2, the total content of gangliosides also was increased in the kidneys of treated animals. The relative percentages of G_{M3} and the other gangliosides, however, were decreased and increased, respectively, by dexamethasone administration (Table 2, Fig. 2).

GLC analyses of the sugar moieties of control and treated tissue further established their identities and showed similar molar ratios for each of the glycosphingolipids of control and dexamethasone-treated kidneys (Table 3).

After acid hydrolysis of control and treated kidneys, the sialic acid moieties of G_{M3} were found to contain both N-acetyl- and N-glycolylneuraminic acid. Although the latter form predominated in both, the relative percentage of N-glycolylneuraminic acid was found to be significantly greater in dexamethasone-treated kidneys (Table 4).

In order to detect additional differences between the glycosphingolipids of control and treated tissue, the long-chain bases and fatty acids of the major glycosphingolipids were examined and compared. As shown in Table 5, sphingosine was the predominant long-chain base of glucosyl-, globotetraosylceramide and G_{M3} in control kidneys. Furthermore, no significant differences were noted in the relative percentages of the long-chain bases of kidneys of control and treated animals (Table 5).

In contrast to these latter findings, dexamethasone treatment appeared to modify the fatty acids of the major kidney glycosphingolipids. As shown in Table 6, the present treatment regimen with this synthetic glucocorticoid significantly altered the individual hydroxy and nonhydroxy fatty acids of glucosyl- and globotetraosylceramide, as well as G_{M3}. As can be seen, dexamethasone administration, in general, decreased the hydroxy and nonhydroxy fatty acid chain length of these major glycosphingolipids, particularly glucosylceramide and G_{M3} (Table 6).

DISCUSSION

Prior studies from a number of laboratories have demonstrated that glucocorticoid hormones, including dexamethasone, could influence the glycerolipid and phospholipid composition of various cell types (27–29). Administration of dexamethasone (2 mg/kg body wt/day)

TABLE 3

Analysis of Sugar Moieties in Neutral Glycosphingolipids and G_{M3} of Control and Dexamethasone-treated Kidneys[a]

	Control		Treated	
	Sugar	Molar ratios	Sugar	Molar ratios
Glucosylceramide	Glc	—	Glc	—
Lactosylceramide	Gal/Glc	1.0:1.0	Gal/Glc	1.1:1.0
Globotriaosylceramide	Gal/Glc	2.1:1.0	Gal/Glc	2.0:1.1
Globotetraosylceramide	Gal/Glc/$GalNA_c$	2.0:1.0:1.1	Gal/Glc/$GalNA_c$	2.1:1.1:1.0
G_{M3}	Gal/Glc/SA	1.0:1.0:1.0	Gal/Glc/SA	0.9:1.1:1.0

[a]Glycosphingolipid classes were separated by TLC, and trimethylsilyl esters prepared and analyzed by GLC, as described in Materials and Methods section.

Glc, glucose; Gal, galactose; $GalNA_c$, N-acetylglucosamine; and SA, sialic acid.

TABLE 4

Relative Percentages of N-acetyl- and N-glycolylneuraminic Acid in G_{M3} of Kidney in Control and Dexamethasone-treated Rats[a]

Form of sialic acid	Control	Dexamethasone-treated
N-acetyl	33.8 ± 1.8	27.0 ± 1.6[b]
N-glycolyl	66.0 ± 1.6	73.1 ± 1.1[b]

[a]Values represent means ± SE of four separate preparations of each group.

[b]$P < 0.05$ compared to control values.

TABLE 5

Percentage of Sphingosine (S) and Phytosphingosine (P) in Glycosphingolipids in Kidneys of Dexamethasone-treated and Control Rats[a]

	Control			Dexamethasone-treated		
Glycosphingolipid	S	P	Other	S	P	Other
G_{M3}	54.1 ± 2.9	25.6 ± 3.1	20.3 ± 2.9	58.3 ± 1.7	20.3 ± 2.1	21.3 ± 0.05
Glucosylceramide	71.1 ± 2.7	21.7 ± 2.2	10.3 ± 1.1	68.1 ± 1.7	21.4 ± 1.4	10.5 ± 1.4
Globotetraosylceramide	72.0 ± 2.8	13.5 ± 0.8	14.5 ± 1.5	71.9 ± 3.5	13.6 ± 1.1	14.8 ± 2.4

[a]Values are expressed as relative percentages and represent means ± SE of four separate preparations (four rats/preparation) of each group.

TABLE 6

Hydroxy and Nonhydroxy Fatty Acid Composition of Major Glycosphingolipids of Kidney in Control and Dexamethasone-treated Rats[a]

	Glucosylceramide				Globotetraosylceramide				G_{M3}			
	Control		Treated		Control		Treated		Control		Treated	
Fatty acids	HFA%	NFA%	HFA%	NFA%	HFA%	NFA%	HFA%	NFA%	HFA%	NFA%	HFA%	NFA%
16:0	14.5	20.4	18.6	19.6	27.0	24.7	28.5	25.2	28.7	32.3	22.7	29.0
16:1	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
18:0	18.2	22.6	24.6	15.5	15.2	6.1	19.4	7.1	24.1	25.4	36.6	31.5
18:1	8.2	11.6	16.1	18.2	6.6	2.8	8.2	3.7	8.9	9.8	9.8	10.1
18:2	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
20:0	9.0	6.2	19.0	20.3	11.5	6.2	14.2	7.2	8.2	8.5	6.2	8.3
20:4	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
22:0	13.5	25.8	9.6	10.6	23.8	7.2	17.5	5.9	9.3	13.5	3.2	8.5
24:0	18.2	3.3	5.3	5.4	8.9	38.5	4.7	36.1	11.2	10.2	5.4	4.0
26:0	16.8	tr	5.0	tr	6.7	3.2	3.2	tr	2.9	tr	2.4	tr

[a]Values represent the mean of four separate preparations. SE were all less than 6% of the mean.

HFA, hydroxy fatty acids; NFA, nonhydroxy fatty acids; tr, trace indicates 2% or less of the total.

to adult male rats for 1 wk, however, failed to alter the total phospholipid content in the kidneys of these animals (29).

Corticosteroids also have been shown to alter the acidic glycosphingolipids of rabbit thymocytes (12). More recently, our laboratory (11) has shown that subcutaneous dexamethasone administration (100 μg/100 g body wt/day) for four days induced changes in the glycosphingolipids of the rat proximal small intestinal mucosa. The present experiments demonstrated, for the first time, that this dexamethasone treatment regimen also results in significant quantitative and qualitative alterations in the neutral and acidic glycosphingolipids of the rat kidney.

Since previous studies have suggested that the glycosphingolipid composition of animals, including rats, is organ-specific (1), it was of particular interest, in the present studies, to compare the effects of dexamethasone treatment on rat kidneys with effects previously seen in the rat small intestine (11). In this regard, dexamethasone significantly increased the total content of ceramide and the neutral glycosphingolipids by ca. 50% in the rat kidney, and moreover, increased the relative percentages of globotriaosyl- and globotetraosylceramide, but reduced the relative percentage of glucosylceramide. In contrast to these current findings, previous administrations of the hormone were not found to affect these lipids in the rat small intestine (11).

In both kidney and small intestine, dexamethasone increased the total content of acidic glycosphingolipids by ca. 50%. In the kidneys, this was associated with a small decrease (10%) in the relative percentage of G_{M3}, but in the small intestine, there was an ca. 15% relative percentage increase (11). Dexamethasone, however, increased the relative percentage of N-glycolylneuraminic acid of G_{M3} in both organs. In the present studies, long-chain bases of the major kidney glycosphingolipids, including G_{M3}, were not found to be influenced by this synthetic glucocorticoid. In earlier studies (11), however, this agent produced decreases in the relative percentage of phytosphingosine in G_{M3}, as well as in glucosyl- and globotriaosylceramide, the major neutral glycolipids of the rat small intestine.

Additionally, relatively minor changes were previously seen in the hydroxy and nonhydroxy fatty acids of small intestinal G_{M3}, secondary to dexamethasone administration (11), but in the present studies, administration of this hormone produced more impressive changes in the relative percentages and chain lengths of these fatty acids in the major kidney neutral and acidic glycolipids. These results deserve further comment.

First, the present results, taken together with our earlier data (11), demonstrate clearly that dexamethasone can influence the glycosphingolipid composition of both the rat kidney and the rat small intestine. In general, however, the alterations induced by this hormone were more extensive in the rat kidney, involving both the neutral and acidic glycosphingolipids. It would appear, therefore, that dexamethasone-induced glycosphingolipid alterations, indeed, are organ-specific in the rat.

Second, treatment with dexamethasone increased the relative percentage of N-glycolylneuraminic acid in G_{M3} of both rat kidney and small intestine. This hormone, however, decreased the relative percentages of phytosphingosine in the major small intestinal glycosphingolipids (11), but in the current experiments, was not found to alter the long-chain bases of these glycolipids in the kidneys. Because the conversion of N-glycolyl- from N-acetylneuraminic acid (30,31) and phytosphingosine from sphinganine (31) involves hydroxylation reactions, it appears that this synthetic glucocorticoid has different affects on these reactions in both organs.

Similarly, intraperitoneal injection of cortisone acetate for 48 hr has been shown previously to decrease the levels of N-glycolylneuraminic acid in G_{M3} of rabbit thymocytes, to not affect its long-chain bases and to increase the chain length of the fatty acids of this ganglioside (12). As noted earlier, in the rat kidney, but not in the intestine (11), dexamethasone produced decreases in the chain length of G_{M3} and the major neutral glycosphingolipids. Taken together, these results suggest that the effects of this hormone on acidic and neutral glycosphingolipids are very complex and are not only organ-specific, but also species-specific.

Third, the mechanism(s) underlying the present quantitative and qualitative alterations in the dexamethasone-induced, rat kidney glycosphingolipids are unclear at this time. Earlier studies concerning sex-hormone effects on rat and mouse kidney glycolipids suggested that these hormones may increase the activities of certain enzymes involved in the biosynthesis of glycosphingolipids (2–5). The increased neutral and acidic glycolipid content seen in the rat kidneys, after dexamethasone administration, may be due to increased rates of lipid biosynthesis, decreased rates of degradation or a combination of both (32). Further studies, therefore, will be necessary to clarify this issue. Regardless of the mechanism(s) involved, however, the present data demonstrate that administration of this synthetic glucocorticoid leads to alterations in both the acidic and neutral glycosphingolipids in rat kidneys.

Finally, it should be noted that, while the physiological significance of these dexamethasone-induced, renal glycosphingolipid alterations remain unclear, prior studies of a number of different organs (1,6–10,33–36), including the rat kidney (1), have suggested that neutral and acidic glycosphingolipids may play a role in several important physiological and pathological processes. For example, addition of exogenous G_{M3} to various cell lines has been shown, in fact, to inhibit cell growth (37). Further studies should elucidate the mechanism(s) responsible for these dexamethasone-induced alterations in the glycolipids of the rat kidney and clarify their functional significance.

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REFERENCES

1. Tomono, Y., Naito, I., and Watanabe, K. (1984) *Biochim. Biophys. Acta 796*, 199–204.
2. Coles, L., and Gray, G.M. (1970) *Biochem. Biophys. Res. Commun. 38*, 520–526.
3. Hay, J.B., and Gray, G.M. (1970) *Biocehim. Biophys. Acta 202*, 566–568.
4. Gray, G.M. (1971) *Biochim. Biophys. Acta 239*, 494–500.

5. McCluer, R.H., and Gross, S.K. (1985) *J. Lipid Res. 26,* 593-599.
6. Berry-Kravis, E., and Dawson, G. (1985) *J. Neurochem. 45,* 1739-1747.
7. Spiegel, S., Fishman, P.H., and Weber, R. (1985) *Science 230,* 1285-1287.
8. Bremer, E.G., Hakomori, S., Bowen-Pope, D.F., Raines, E., and Ross, R. (1984) *J. Biol. Chem. 259,* 6818-6825.
9. Spiegel, S., Yamada, K.M., Hom, B.E., Moss, J., and Fishman, P.H. (1986) *J. Cell Biol. 102,* 1898-1906.
10. Spiegel, S., Handler, J.S., and Fishman, P.H. (1986) *J. Biol. Chem. 261,* 15755-15760.
11. Dahiya, R., and Brasitus, T.A. (1987) *Biochim. Biophys. Acta 922,* 118-124.
12. Iwamori, M., Kiguchi, K., Kanno, J., Kitagawa, M., and Nagai, Y. (1986) *Biochemistry 25,* 889-896.
13. Glickman, R.M., and Bouhours, J.F. (1976) *Biochim. Biophys. Acta 424,* 17-25.
14. Suzuki, A., Kundu, S.K., and Marcus, D.M. (1980) *J. Lipid Res. 21,* 473-477.
15. Folch, J., Lees, M., and Sloane-Stanley, G.A. (1957) *J. Biol. Chem. 226,* 497-509.
16. Williams, M.A., and McCluer, R.H. (1980) *J. Neurochem. 35,* 266-269.
17. Saito, T., and Hakomori, S.I. (1971) *J. Lipid Res. 12,* 257-259.
18. Mullin, B.R., Poore, C.M.B., and Ruppe, B.H. (1984) *J. Chromatogr. 305, 512-513.*
19. *Siddiqui, B., and Hakomori, S.F. (1973) Biochim. Biophys. Acta 330,* 147-155.
20. Leeden, R., Salsman, K., and Cabrera, M. (1968) *Biochemistry 7,* 2287-2295.
21. Reinhold, V.N. (1972) *Methods Enzymol. 25,* 244-248.
22. Esselman, W.J., Laine, R.A., and Sweeley, C.C. (1972) *Methods Enzymol. 28,* 140-156.
23. Sweeley, C.C., and Moscatelli, E.A. (1959) *J. Lipid Res. 1,* 40-47.
24. Bouhours, J.F. (1979) *J. Chromatogr. 169,* 462-465.
25. Bouhours, J.F., and Glickman, R.M. (1977) *Biochim. Biophys. Acta 487,* 51-60.
26. Carter, H.E., and Gaver, R.C. (1967) *J. Lipid Res. 8,* 391-395.
27. Murray, D.K., Ruhmann-Wennhold, A., and Nelson, D.H. (1979) *Endocrinology 105,* 774-777.
28. Mangiapane, E.H., and Brindley, D.N. (1986) *Biochem. J. 233,* 151-160.
29. Young, S.L., and Silbajoris, R. (1986) *J. Appl. Physiol. 60,* 1665-1672.
30. Schoop, H.J., Schaver, R., and Faillard, H. (1969) *Hoppe-Seyler's Z. Physiol. Chem. 350,* 155-162.
31. Bouhours, D., and Bouhours, J.F.)1983) *J. Biol. Chem. 258,* 299-304.
32. Dahiya, R., and Brasitus, T.A. (1987) *Biochim. Biophys. Acta 918,* 230-235.
33. Fishman, P.H. (1986) *Chem. Phys. Lipids 42,* 137-151.
34. Merrill, A.H. Jr., Sereni, A.M., Stevens, V.L., Hannun, Y.A., Bell, R.M., and Kinkade, J.M. Jr. (1986) *J. Biol. Chem. 261,* 12610-12615.
35. Kreutter, D., Kim, J.Y.H., Goldenring, J.R., Rasmussen, H., Ukomadu, C., Delorenzo, R.J., and Yu, R.K. (1987) *J. Biol. Chem. 262,* 1633-1637.
36. Dahiya, R., Dudeja, P.K., and Brasitus, T.A. (1987) *Cancer Res. 47,* 1031-1035.
37. Bremer, E.G., and Hakomori, S.I. (1982) *Biochem. Biophys. Res. Commun. 106,* 711-712.

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Characterization of Skin-surface Lipids from the Monkey *(Macaca fascicularis)*

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Skin-surface lipids from the monkey *Macaca fascicularis* are composed of sterol esters (38%), cholesterol (4%) and two types of wax diesters, identified as Type II (IIa and IIb, 17% and 40%, respectively). Type IIa contained diesters of 1,2-alkanediols esterified with two molecules of long-chain (C_{14}–C_{34}) fatty acids having straight and branched chains. In the diesters IIa, fatty acids shorter than C_{19} predominated in position 1, and fatty acids longer than C_{20} predominated in position 2. Type IIb contained diesters of 1,2-alkanediols esterified with C_4 and C_5 branched-chain fatty acids (predominantly isovaleric acid) at position 1 and long-chain (C_{14}–C_{27}) acids, having straight and branched chains, at position 2. The short-chain acids were converted to 2-nitrophenylhydrazides and analyzed by high-performance liquid chromatography (HPLC). Ammonia chemical ionization (CI)–gas chromatography (GC)–mass spectrometry (MS) resolved the intact diesters IIb into 12 peaks corresponding to molecular weights ranging from 597 to 748, and showed that the molecular species, such as C_{21}-C_{16}-C_5 (diol, fatty acid in position 2, fatty acid in position 1), C_{22}-C_{16}-C_5 and C_{23}-C_{16}-C_5, were prevalent. The fatty acids from both diesters were mostly (>98%) saturated. The 1,2-alkanediols from both diesters consisted of C_{16}-C_{26} saturated straight- and branched-chain components. The acyl groups of sterol esters contained 86% C_{14}-C_{34} branched-chain acids. The unsaturated fatty acids (5.4%) belonged to a straight-chain monoenoic series having extremely long chains (C_{18}-C_{34}). The branched-chain structures in the fatty acids and diols were iso and anteiso. These results show the species-specific profile for the skin-surface lipid synthesis.
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Besides their physiological functions, skin-surface lipids are thought to play a significant role in excreting lipophilic compounds from the body. Wolff (1) analyzed halogenated hydrocarbons in human skin-surface lipids for estimation of human body burden. For assessment of human exposure to environmental pollutants, using skin lipids as a specimen, animal experiments are required.

Skin-surface lipids contain unusual lipids that are not commonly found in the internal tissues, and there is a wide variation between species, both in the classes of lipids present and in the structures of the aliphatic moieties (2–12). Human skin-surface lipids differ markedly from those of animals; they contain triacylglycerols and squalene as major constituents, whereas those of other animals consist primarily of a variety of wax mono- and diesters (9,10). It, therefore, was of interest to examine the skin-surface lipids of primates. An early investigation of primates used thin-layer chromatography (TLC) for the study of the surface lipids of the chimpanzee and baboon (2). To evaluate the use of animal models, detailed surface-lipid characterization is required to decide whether the origin and biosynthesis of the lipids is comparable with those of humans.

In the present study, the monkey *Macaca fascicularis* was chosen to determine in detail the composition and structures of its skin-surface lipids. On the basis of these results, lipogenesis in the sebaceous gland was deduced. In addition, it was shown that the derivatization to 2-nitrophenylhydrazide was useful for the analysis of short-chain fatty acids in diesters, and that ammonia chemical ionization–gas chromatography–mass spectrometry (CI-GC-MS) was suitable for the molecular species analysis of the diesters.

MATERIALS AND METHODS

Collection of the skin-surface lipids. The skin-surface lipids were collected from the backs of three male monkeys *(M. fascicularis).* After being shaven, the backs (10 × 10 cm) were wiped with gauze (10 × 10 cm) soaked in acetone/hexane (1:1, v/v), that had been exhaustively extracted with the solvent. The lipids were eluted from the gauze with acetone/hexane (1:1, v/v) on a sintered glass filter. The solvent was evaporated, yielding 6–15 mg of lipids per animal. Because preliminary analysis showed no significant difference in the composition of lipid classes and the constituent fatty acids between individual animals, the collection was repeated 3–4 times (once a day at intervals of 3–4 days); the lipid samples were combined and used for determination of chemical structures. The results were an average of three separate experiments.

Separation of lipid classes. The total-surface lipids were fractionated on a 0.25-mm-thick silica gel plate (Merck $60F_{254}$), using hexane/benzene (2:3, v/v). The lipid bands were located with iodine vapor and separately scraped from the plate, after which the lipids were eluted with diethyl ether. The sterol ester fraction was rechromatographed on magnesium hydroxide TLC with hexane/ethyl acetate (100:1, v/v) (12). This procedure separated the sterol esters from a small amount of wax monoesters, as described by Stewart and Downing (12). The bands were visualized under UV-light after spraying with 2′,7′-dichlorofluorescein in methanol (1 mg/ml).

Sterol esters. The sterol esters were saponified in 1 ml 0.5 N NaOH in 95% methanol/benzene (4:1, v/v) at 60 C for one hr. After acidification with 1 N HCl, the products were recovered in hexane, and then treated with 1 ml benzene/5% HCl in methanol (1:2, v/v) at 100 C for 30 min. After adding water, the products were extracted with diethyl ether, and then fractionated by TLC (10 × 10 cm).

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Abbreviations: FAS, fatty acid synthetase; TMS, trimethylsilyl; CI, chemical ionization; CI-GC-MS, chemical ionization-gas chromatography-mass spectrometry; ECL, equivalent chain length; EI, electron impact; FCL, fractional chain length; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; TIC, total ion monitoring; TLC, thin-layer chromatography.

After successive development with hexane/benzene (2:3, v/v, to 10 cm), followed by benzene/ethyl acetate (1:1, v/v, to 4 cm), the two bands were scraped from the plate, and the fatty acid methyl esters and free sterols were eluted from the adsorbent with diethyl ether and chloroform/methanol (2:1, v/v), respectively. The free sterols separated by TLC were converted to their TMS-derivatives by treatment with N,*O*-bis(trimethylsilyl)acetamide at 60 C for one hr.

An aliquot of the fatty acid methyl esters was converted to pyrrolidides by heating with 1 ml pyrrolidine/acetic acid (10:1, v/v) at 100 C for one hr. After adding 3 N HCl (3 ml), the products were extracted with hexane. The extract was successively washed with 3 N HCl and then with water, after which it was dried over anhydrous magnesium sulfate. After removing the solvent under a stream of nitrogen, the residue was subjected to GC.

The saturated and monounsaturated fatty acid methyl esters were separated by argentation-TLC using hexane/diethyl ether (9:1). The plates were prepared by immersion of precoated silica gel plates in a solution of 4% silver nitrate in acetone/water (9:1, v/v). The methyl esters were located with 2′,7′-dichlorofluorescein and recovered in ether. The double bonds of the unsaturated fatty acids were cleaved by Lemieux-von Rudloff oxidation (13), as described in the next section. After 60 hr, the fatty acids were extracted and treated with diazomethane. Hydrogenation was carried out by bubbling hydrogen through the methanol solution (0.5 ml) of the unsaturated fraction in the presence of 5% Pd-$CaCO_3$ (1–2 mg). The structure of each fatty acid was assigned from the mass spectra of methyl ester and/or pyrrolidide according to established data (14,15).

Wax diesters. Each of the isolated wax diester fractions (IIa and IIb) was saponified and then processed in the same manner as the sterol esters.

The wax diols were converted to their isopropylidene derivatives by treatment with 1 ml acetone and 2 μl 70% perchloric acid at room temperature. After 30 min, the mixture was made alkaline with ammonium hydroxide and evaporated to dryness under a nitrogen stream. The products were extracted into hexane. Traces of polar material were removed by preparative TLC and developed in hexane/benzene (2:3, v/v).

Aliquots of the diols were oxidized by the Lemieux and von Rudloff method (13). To the solution of alkanediols in 0.5 ml tert-butanol were added 0.2 ml 0.1 N Na_2CO_3, 0.2 ml 0.5 N $NaIO_4$ and 0.1 ml 8 mM $KMnO_4$. After 24 hr, the fatty acids formed were extracted with hexane from the acidified reaction mixture. The extract was washed with water, dried with anhydrous magnesium sulfate and evaporated to dryness. The fatty acids were converted to methyl esters by treatment with diazomethane for GC analysis.

A portion of the diesters IIb was treated with 0.2 ml pyrrolidine and 20 μl acetic acid, and the component fatty acids were converted directly to pyrrolidides. After heating at 100 C for two hr, an aliquot was submitted to GC–MS analysis. Authentic samples of the short-chain fatty acid pyrrolidides were prepared from their ethyl or propyl esters. Their mass spectra were as follows: 2-methylpropanoyl pyrrolidine, m/z (rel intensity): 141 (M^+, 44), 126 (15), 112 (4), 98 (100); 2-methylbutanoyl pyrrolidine, 155 (M^+, 36), 140 (38), 127 (100), 112 (8), 98 (94); 3-methylbutanoyl pyrrolidine, 155 (M^+, 29), 140 (6), 113 (100), 98 (21). Wax diesters IIb (0.5 mg) were saponified with 0.1 ml 2.5 M KOH in 85% ethanol at 90 C for one hr. The free fatty acids were converted to the 2-nitrophenylhydrazides by treatment with 2-nitrophenylhydrazine hydrochloride (2-NPH · HCl) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1-EDC · HCl) as a coupling agent (16).

Lipase hydrolysis. Wax diesters IIa (6 mg/2 ml) were emulsified, and then hydrolyzed with 25 μl of lipase solution (*Rhizopus arrhizus,* 10 mg/ml, Boehringer-Mannheim, Mannheim, FRG) at 39 C, as described by Schmid and Schmid (17). After 1.5 hr, the products were extracted and analyzed by TLC using hexane/diethyl ether/acetic acid (80:20:1, v/v/v), as described (17). The fractionation was carried out by TLC using benzene/ethyl acetate (5:2, v/v). Unhydrolyzed diesters ($R_f \sim 0.98$), 2-acyl diols ($R_f \sim 0.8$), and free fatty acids ($R_f \sim 0.5$) were isolated. During this process, free diols ($R_f \sim 0.2$) were not detected. Free fatty acids or 2-acyldiols were treated with benzene/5% HCl in methanol (1:2, v/v) at 100 C for two hr. Methyl esters from 2-acyl diols were purified by TLC in hexane/benzene (4:6, v/v) before analysis by GC.

Dieters IIb (6.0 mg) also were hydrolyzed with 50 μl of lipase. After four hr, an aliquot of the reaction mixture was treated with the 2-NPH and 1-EDC solution, as described by Miwa and Yamamoto (18), and the free fatty acids were converted to 2-nitrophenylhydrazides for high-performance liquid chromatography (HPLC) analyses. The hydrolysis products were extracted from the remainder and fractionated by TLC in the same manner as previously described. No free fatty acids were detected by TLC. The 2-acyldiols or unhydrolyzed diesters were hydrolyzed with alkali, and the constituted fatty acids were converted to methyl esters or 2-nitrophenylhydrazides.

GC. GC analyses were performed with a Shimadzu GC 9A gas chromatograph equipped with a flame-ionization detector and a 30 m × 0.25 mm DB-5 column (J & W Scientific Inc., Folsom, CA). Helium at 1.3 kg/cm² pressure was used as the carrier gas. The column was programmed at 4 C/min at 200–320 C for the fatty acid methyl esters, at 250–320 C for the fatty acid pyrrolidide, at 220–300 C for the isopropylidene derivatives of alkanediols, and at 300–320 C for the TMS-derivatives of sterols. For measuring the equivalent chain lengths (ECL), appropriate isothermal conditions were used. The sample was introduced by the split technique with a 50:1 split ratio.

GC–MS and MS. EI–GC–MS analyses were performed with a JEOL DX-300 mass spectrometer interfaced to a GCG06 gas chromatograph and to a JMA–5000 mass data system. The column was a 30 m × 0.25 mm DB-5 capillary terminating inside the ion source. Operating conditions for the column were the same as mentioned above. The split ratio was 10:1. Mass spectra were obtained at a scan speed of 1 s decade^{-1}, an accelerating voltage of 70 eV and an ion source temperature of 250 C.

For analyses of the intact diesters IIb, the compounds were introduced by direct insertion probe or by GC inlet via a glass jet separator. The CBP1 column (4 m × 0.53 mm, Shimadzu Co. Ltd., Kyoto, Japan) was programmed at 270–320 C at 8 C/min. The injector and jet separator were held at 300 C. The ion source temperatures

were 200 C for the direct inlet system and 270 C for the GC inlet system. CI spectra were obtained at 250 eV.

HPLC. HPLC analyses were carried out using a Shimadzu Model LC-3A liquid chromatograph. The column was Zorbax ODS (5 μm, 4.6 mm × 25 cm, Du Pont Co., Wilmington, DE). The eluents were acetonitrile/water (40:60, v/v) for short-chain (C_2–C_6) 2-nitrophenylhydrazide derivatives and (95:5, v/v) for the long-chain (C_{14}–C_{20}) derivatives; they were adjusted to pH 4.5 with 0.1 N HCl, as described by Miwa and Yamamoto (16,18). The column effluent was monitored at 390 nm at a flow rate of 1.2 ml/min.

RESULTS

Separation of lipid classes. TLC analysis showed the presence of four major neutral lipid constituents. They were ultimately identified as sterol esters, free cholesterols and two types (IIa and IIb) of Type II wax diesters (Fig. 1). One (IIa, $R_f \sim 0.57$) migrated like a Type II diester (diacyl alkanediols) from the rat skin (4) on TLC, and the other (IIb) showed a lower mobility ($R_f \sim 0.48$). The contents, based on their weight, were 38% sterol esters, 17% IIa, 40% IIb and 4% free cholesterols.

Sterol esters. After magnesium hydroxide TLC, the sterol ester fraction showed one major spot, corresponding to the sterol esters, and one minor spot, corresponding to the wax monoester standard. However, no detailed examination of the latter component was carried out, because the content was extremely low. After saponification, followed by methylation, the purified sterol esters showed two spots on TLC due to fatty acid methyl esters and sterols. GC and GC–MS of the TMS-derivatives of the sterol fraction yielded only one peak coinciding with the cholesterol-TMS standard.

The fatty acid methyl esters were examined by argentation-TLC and by capillary-GC, before and after hydrogenation with H_2/Pd-$CaCO_3$. The saturated fatty acids were grouped into three homologous series according to their fractional chain length (FCL) (ECL − carbon number) values. The carbon skeletal types of the series having FCL values of 0.00, 0.64 and 0.74 were assigned to straight, iso and anteiso, respectively, from GC–MS analyses of the pyrrolidides (shorter chains than C_{27}) and the methyl esters. The chain lengths of the fatty acids showed a wide distribution, ranging from C_{14}–C_{34}, with a maximum around C_{20}–C_{21} (Table 1).

The content of unsaturated fatty acids was extremely low (total 5.4%). They were predominantly a monoenoic series, having C_{18}–C_{34} chains. Hydrogenation with H_2/Pd-$CaCO_3$ showed that 99% of them had straight chains. Determination of the double-bond position for each peak was not carried out, because their amounts were low and pyrrolidides longer than C_{28} were unsuitable for GC (or GC–MS) analysis. However, Lemiuex-von Rudloff oxidation of the total unsaturated fatty acids produced heptanoic and nonanoic acids, and a series of odd-chain C_{21}–C_{27} dicarboxylic acids (predominantly C_{23} and C_{25}), showing that most of these monoenoic acids belonged to the n-7 or the n-9 series.

Wax diesters IIa. After saponification and methylation, diesters IIa gave two spots on TLC, due to fatty acid methyl esters and a polar component.

The polar component was converted to the isopropylidene derivative, showing the presence of a vicinal-diol (-CH(OH)-CH(OH)-) group. Only one spot, corresponding to saturated diol isopropylidene derivatives, was detected by argentation-TLC. When the mass spectra of the free diols and the corresponding isopropylidene derivatives were compared, the characteristic ions due to cleavage of a bond between the two hydroxy groups on

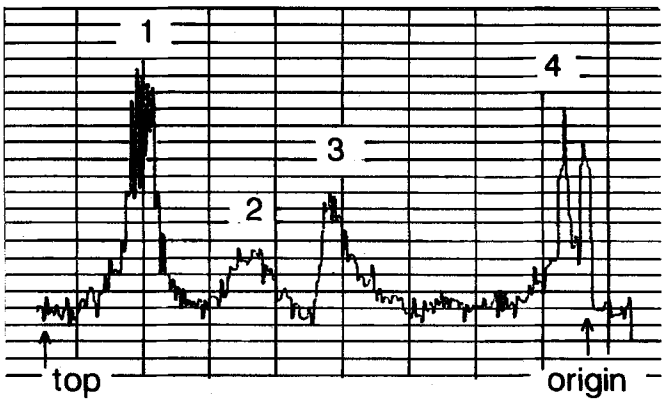

FIG. 1. Densitometer scan of a thin-layer chromatogram of monkey skin-surface lipids. The chromatogram was developed with hexane/benzene (2:3), sprayed with 50% sulfuric acid and charred at 220 C. Numbers above the chromatogram indicate: 1, sterol esters; 2, wax diesters IIa; 3, wax diesters IIb; 4, cholesterol.

TABLE 1

Constituent Fatty Acids (mol %) of Sterol Esters from Monkey Skin-surface Lipids

Number of C-atoms	Straight-chain (14.34%)				Branched-chain (85.66%)		
	Saturated	Monoenoic			Iso		Anteiso
16	0.90		—[a]	—	—		
17		0.10				0.14	0.08
18	1.05		0.08[b,o]	1.41[c] 0.14[d]	0.09		
19		0.13				3.35	0.22
20	2.59		—	—	7.74		
21		0.17				26.00	12.19
22	1.51		0.05[e]	0.09[f]	9.24		
23		0.11				8.29	6.85
24	0.94		—	0.16[g]	1.34		
25		0.08				2.85	1.73
26	0.48		—	—	0.79		
27		0.03				1.23	1.01
28	0.28		—	0.29[h]	0.30		
29		—				0.54	0.42
30	0.29		0.06[i]	0.89[j]	0.18		
31		—				0.36	0.29
32	0.22		0.22[k]	1.15[l]	0.10		
33		—				0.14	0.16
34	0.03		0.35[m]	0.44[n]	—		
Totals	8.29	0.62	5.43		19.81	42.90	22.95

[a]Proportions less than 0.03% are designated (—).

[b-n]ECL of each peaks is as follows: b, 17.72; c, 17.77; d, 17.82; e, 21.78; f, 21.85; g, 23.88; h, 27.92; i, 29.86; j, 29.93; k, 31.84; l, 31.90; m, 33.79; n, 33.86.

[o]$C_{18:2}$.

the spectrum of the free diols were observed at $[M - 31]^+$, but not at $[M - 45]^+$. This result indicates that the two hydroxy groups were located at positions 1 and 2 of the alkanediols ($R-CH(OH)-CH_2(OH)$). Furthermore, the diols were chemically cleaved by Lemiuex-von Rudloff oxidation and the resulting fatty acids were esterified. GC analysis of these methyl esters showed a chain-length distribution pattern similar to that of the original diols, but with the corresponding peaks containing one less carbon atom, thus confirming the 1,2-structure in the diols. In addition, GC–MS analysis of these methyl esters indicated that the alkyl-chain structures of the original diols were straight, iso and anteiso. The diols contained 74.8% branched and 25.2% straight components, ranging from C_{16}-C_{26}, with a maximum around C_{20}-C_{22} (Table 2).

The fatty acids consisted of straight-, iso- and anteiso-chain components, ranging from C_{14}-C_{34} (Table 3). Of the straight-chain components, the largest peak was C_{16}. In contrast, branched-chain components gave a two-cluster profile, with a maxima around C_{16}-C_{17} and C_{21}-C_{22}.

The result of the lipase hydrolysis showed nonrandom distribution of the fatty acids between positions 1 and 2 of the 1,2-diols (Table 3). Although the straight-chain C_{16} species was the most abundant constituent in both positions, the shorter chain acids ($<C_{20}$) were prevalent in position 1 and the longer chain acids ($\geq C_{20}$) were prevalent in position 2. In particular, this tendency was observed in the branched-chain components.

Wax diesters IIb. Saponification, followed by methylation of diesters IIb, yielded fatty acid methyl esters and diols. The composition of the diols from this fraction was similar to that from diesters IIa. The long-chain fatty acids had the same types of carbon skeletons as those from IIa, and the straight-chain components were more abundant (69.9%) than those from diesters IIa. The carbon chain lengths (C_{14}-C_{26}) were shorter than those from diesters IIa (Table 4).

Dog diesters, which had a lower mobility than Type II diesters, contained isovaleric acid, together with long-chain fatty acids (6). The diesters IIb were examined for the presence of short-chain fatty acids by conversion to their pyrrolidides and 2-nitrophenylhydrazides. Because saponified samples can be derivatized into hydrazides without extraction (16), short-chain acids can be detected quantitatively as their hydrazides. HPLC analysis of the hydrazides from the hydrolysate showed one major peak, corresponding to isovaleric acid hydrazide, and two minor peaks, corresponding to 2-methylbutyric acid and isobutyric acid hydrazides (Fig. 2A). These short-chain acids were identified by GC–MS as their pyrrolidides (Fig. 2B), in comparison with authentic samples.

Figure 3 shows mass spectra for the intact diesters IIb, measured via direct probe under CI conditions. A cluster of ions, m/z 623–679, were observed in isobutane CI (Fig. 3A), and m/z 640–696, in ammonia CI (Fig. 3B). They correspond to $[M+H]^+$ and $[M+NH_4]^+$, respectively, if one molecule of the diesters IIb contains equimolar of short- and long-chain fatty acids and the molecular weight is calculated on the basis of the chain-length composition of the constituents.

Lipase hydrolysis of IIb yielded exclusively short-chain (C_4 and C_5) acids; upon HPLC of the hydrazides of the lipase hydrolysate, only short-chain (C_4-C_5) acids were detected (Table 4). On the contrary, the 2-acyldiol fraction contained exclusively long-chain acids with compositions consistent with those of the long-chain acids from the original IIb. Therefore, it appears that position 1 of 1,2-diols was esterified with one molecule of a short-chain (C_4-C_5) fatty acid, and that position 2 was esterified with one molecule of a long-chain (C_{14}-C_{26}) fatty acid. However, lipase hydrolysis yielded a low proportion of isovaleric acid. Since lipase hydrolysis of IIb was slow and the unhydrolyzed IIb (ca. 70% of the original IIb) contained exclusively isovaleric acids as the short-chain constituent, it is possible that the ester bond of isovaleric acid is resistant to hydrolysis by *Rhizopus arrhizus* lipase. The long-chain fatty acid composition of the unhydrolyzed IIb was identical with that of the original IIb.

TABLE 2

Constituent 1,2-Alkanediols (mol %) of Wax Diesters IIa and IIb from Monkey Skin-surface Lipids

Number of carbon atoms	IIa				IIb			
	Straight-chain (25.21%)		Branched-chain (74.80%)		Straight-chain (16.51%)		Branched-chain (83.50%)	
	Saturated	Monoenoic	Iso	Anteiso	Saturated	Monoenoic	Iso	Anteiso
16	3.17	—[a]	0.24		0.49	—	0.07	
17	0.15		1.49	0.11	0.04		0.23	0.02
18	3.04	—	1.18		1.04	—	0.70	
19	0.29		4.19	0.19	0.25		3.60	0.14
20	12.58	—	2.63		10.35	—	3.23	
21	0.94		25.07	5.80	0.76		29.78	7.38
22	4.59	0.11	14.14		2.74	0.55	17.33	
23	0.15		10.23	6.25	0.11		10.74	7.36
24	0.14	—	2.32		0.18	—	2.09	
25	—		0.47	0.44	—		0.38	0.42
26	0.04	—	0.06		—	—	0.03	
Totals	23.57 1.53	0.11	20.57 41.45	12.78	14.80 1.16	0.55	25.45 44.73	15.33

[a]Proportions less than 0.01% are designated (—).

TABLE 3

Constituent Fatty Acids (mol %) of Wax Diesters IIa from Monkey Skin-surface Lipids

Number of carbon atoms	Total				Position 1				Position 2			
	Straight-chain (50.59%)		Branched-chain (49.41%)		Straight-chain (53.85%)		Branched-chain (46.17%)		Straight-chain (47.97%)		Branched-chain (51.87%)	
	Saturated (48.64%)	Monoenoic (1.95%)	Iso (41.36%)	Anteiso (8.05%)	Saturated (52.08%)	Monoenoic (1.77%)	Iso (37.68%)	Anteiso (8.49%)	Saturated (47.16%)	Monoenoic (0.81%)	Iso (43.80%)	Anteiso (8.07%)
14	0.34	—[a] —	0.13		0.49	— —	0.24		0.37	— —	0.17	
15	0.31		1.17	0.23	0.53		4.35	0.72	0.37		0.37	0.22
16	31.97	— —	0.85		33.75	— —	2.19		35.09	— —	0.87	
17	0.68	0.09[b]	5.32	1.14	1.30	0.05[b]	10.25	2.39	0.12		1.85	0.65
18	5.72	0.55 0.18	0.85		9.30	0.72 0.22	1.20		0.45	0.11 —	0.09	
19	0.18		2.17	0.35	0.20		2.42	0.44	0.11		0.63	0.10
20	4.80	0.10[c] 0.04[d]	1.89		2.23	— —	0.96		6.14	— —	2.25	
21	0.29		13.01	2.51	0.13		3.65	0.87	0.44		19.61	3.63
22	2.00	0.18 0.17	4.75		0.33	— —	1.66		2.18	0.13 0.11	7.06	
23	0.09		5.23	1.62	0.09		3.40	1.03	0.08		5.88	1.75
24	0.88	— 0.05	0.98		0.91	— 0.03	1.07		0.68	— —	0.95	
25	0.08		2.16	0.95	0.08		2.26	1.08	0.07		1.91	0.89
26	0.47	— —	0.58		0.59	— —	0.79		0.38	— —	0.55	
27	0.02		0.87	0.61	0.04		1.15	0.92	0.02		0.67	0.52
28	0.26	— 0.05	0.23		0.41	— 0.07	0.37		0.33	— 0.21	0.20	
29	—		0.43	0.28	—		0.64	0.48	—		0.24	0.02
30	0.33	— 0.13	0.17		0.48	0.02 0.17	0.29		0.20	— 0.05	0.13	
31	—		0.34	0.24	—		0.50	0.37	—		0.22	0.18
32	0.21	0.04 0.26	0.09		0.22	0.07 0.25	0.14		0.13	0.02 0.10	0.07	
33	—		0.13	0.13	—		0.15	0.19	—		0.08	0.11
34	—	0.11 0.12	—		—	0.10 0.07	—			0.04 0.04	—	
	Total	Straight-	Branched-chain		Total	Straight-	Branched-chain		Total	Straight-	Branched-chain	
<20	52.23	(40.02)	(12.21)		70.76	(46.56)	(24.20)		41.57	(36.62)	(4.95)	
≥20	47.77	(10.57)	(37.20)		29.26	(7.29)	(21.97)		58.27	(11.35)	(46.92)	

[a]Proportions less than 0.01% are designated (—).

[b]$C_{18:2}$.

[c]ECL is 19.77.

[d]ECL is 19.84.

TABLE 4

Constituent Fatty Acids (mol%) of Wax Diesters IIb from Monkey Skin-surface Lipids

Number of carbon atoms	Total				Position 1		Position 2			
	Straight-chain		Branched-chain		Branched-chain		Straight-chain		Branched-chain	
	Saturated	Monoenoic	Iso	Anteiso	Iso	Anteiso	Saturated	Monoenoic	Iso	Anteiso
4	—		2.00		6.00		—		—	
5		—		87.90	10.10	55.50	38.50		—		—	—
14	0.49	—[a] —	0.18			—			0.56	— —	—	
15		0.57		2.28	0.29		—	—		0.71		2.60	0.45
16	63.97	— —	2.19			—			64.53	— —	2.69	
17		0.21	0.07[b]	4.99	1.70		—	—		0.26		5.10	2.09
18	0.48	0.34 0.14	0.14			—			0.66	0.19 0.08	0.18		
19		0.04		0.57	0.12		—	—		0.06		0.49	0.12
20	1.77	0.16 0.10	1.41			—			1.67	— —	1.26		
21		0.11		8.45	1.95		—	—		0.11		7.41	1.74
22	0.48	0.16 0.38	2.75			—			0.47	— 0.04	2.51		
23		0.01		1.51	0.59		—	—		—		1.63	0.59
24	0.15	— 0.05	0.18			—			0.16	— —	0.26		
25		0.02		0.38	0.17		—	—		—		0.49	0.23
26	0.09	— —	0.09			—			0.08	— —	0.12		
27		—		0.09	0.06		—	—		—		0.15	0.12
Totals	67.42	0.96 1.52	6.94	18.27	4.88				68.13	1.14 0.31	7.02	17.85	5.34

[a]Proportions less than 0.01% are designated (—).

[b]$C_{18:2}$.

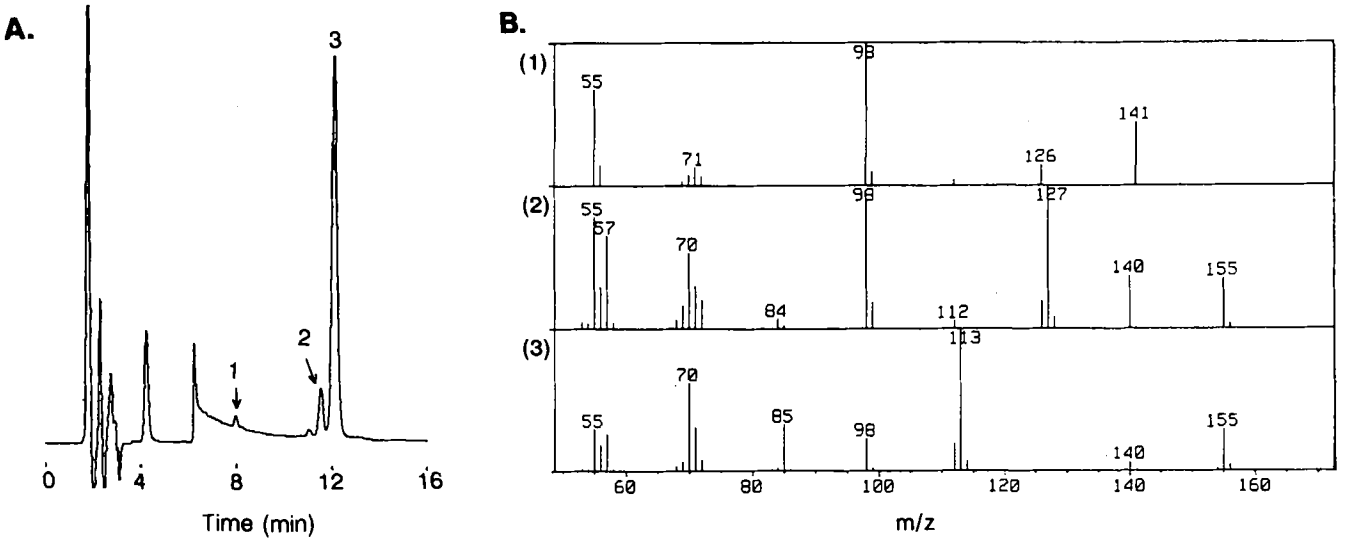

FIG. 2. Short-chain fatty acids of diester IIb. (A) High-performance liquid chromatogram of the 2-nitrophenylhydrazides of the short-chain fatty acids. The peaks 1-3 correspond to isobutyric, 2-methylbutyric and isovaleric acid pyrrolidides, respectively. The other peaks originate from the excess reagents or the reaction byproducts that were observed in a blank run. (B) Mass spectra for the short-chain fatty acid pyrrolidides. Spectra (1)-(3) represent isobutyric, 2-methylbutyric and isovaleric acid pyrrolidides. Each spectrum was identical with that of the standard.

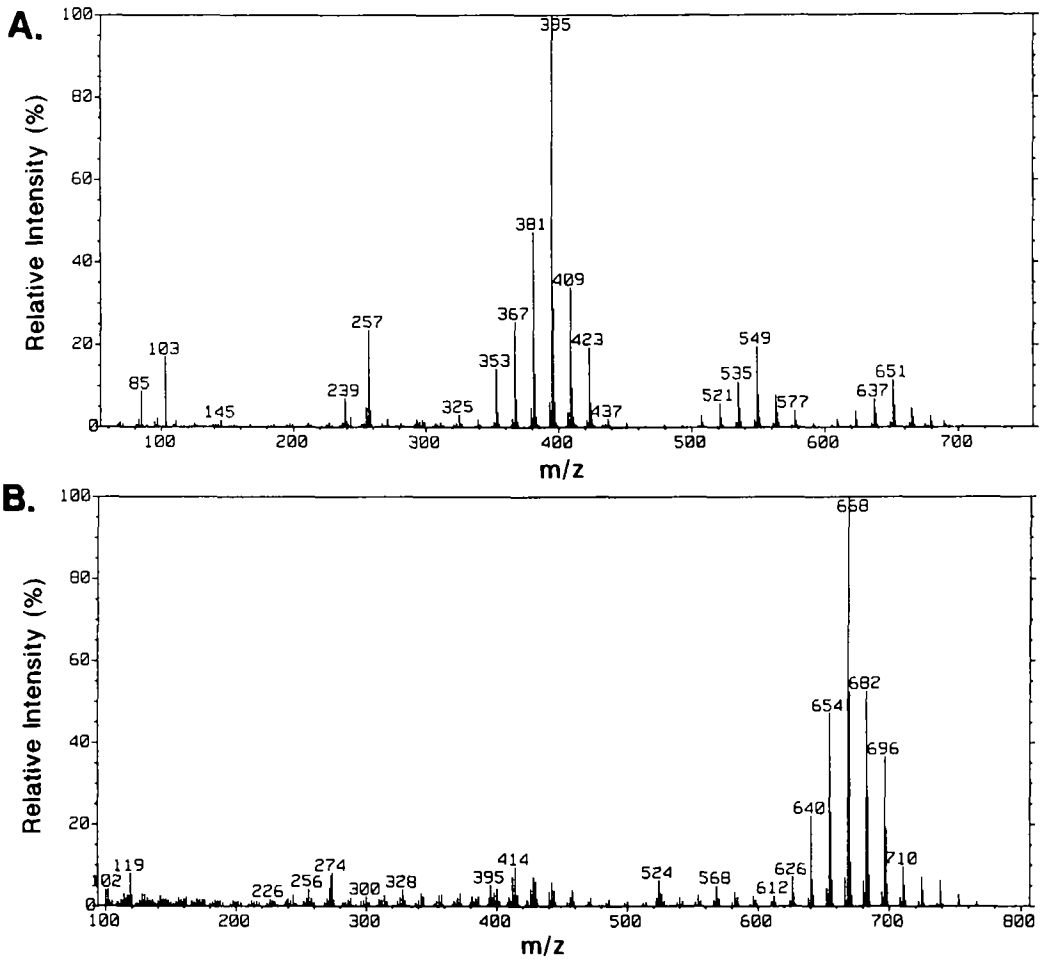

FIG. 3. Mass spectra for intact diesters IIb introduced by direct probe: (A) Isobutane chemical ionization; (B) ammonia chemical ionization.

Furthermore, ammonia CI-GC-MS provided additional evidence to confirm the proposed structure. The spectrum of the largest peak showed that the three diagnostic ions corresponded to $[M+NH_4]^+$ of M+18, $[MH-C_4H_9COOH]^+$ of M − 102, and $[MH-C_{15}H_{31}COOH]^+$ of M − 256 (Fig. 4A).

As shown in Figure 4B, the total ion monitoring (TIC) did not give a complete resolution of the peaks, probably owing to the components having odd carbon numbers and/or branched chains. However, by monitoring the $[M+18]^+$ ions, the diesters were resolved into 12 peaks, because of the carbon numbers. The composition calculated from the peak areas (Table 5) was close to the theoretical values calculated from the components. The average molecular weight based on the peak area was 665, and that on the components was 668.

Mass chromatograms monitoring the $[MH-102]^+$ ions (m/z 507-647) depicted the same pattern as those for the $[M+18]^+$ ions, showing that all peaks had C_5 acid as a major short-chain constituent. Therefore, the $[MH-RCOOH]^+$ ion, that resulted with the loss of the long-chain fatty acid and corresponds to [diol + short-chain acid − 34], can provide the information regarding the diol component. Moreover, compared with the corresponding $[M+18]^+$ ion, the long-chain fatty acid component can be deduced. Table 5 shows the major molecular

TABLE 5

Composition of Diesters IIb Ascertained by GC-MS Under Ammonia CI Condition

M+18 (m/z)	%[a]	Molecular species[b]
612	0.5	16-17-5, 17-16-5
626	2.7	18-16-5
640	5.8	19-16-5, (20-15-5, 21-14-5)[c]
654	14.6	20-16-5, (21-15-5)
668	23.0	21-16-5
682	17.1	22-16-5
696	14.8	23-16-5
710	6.2	24-16-5, (19-21-5, 20-20-5)
724	4.9	20-21-5, 21-20-5, 25-16-5
738	5.4	21-21-5, (20-22-5)
752	3.2	21-22-5, 22-21-5
766	2.0	21-23-5, 23-21-5

[a]Values were calculated from the mass-chromatograms monitoring $(M+18)^+$ ions.

[b]Molecules are shown as the diol-fatty acid in position 2-fatty acid in position 1. 16-17-5 represents $C_{14}H_{29}CH(OCOC_{16}H_{33})CH_2(OCOC_4H_9)$.

[c]The species in parentheses show minor components.

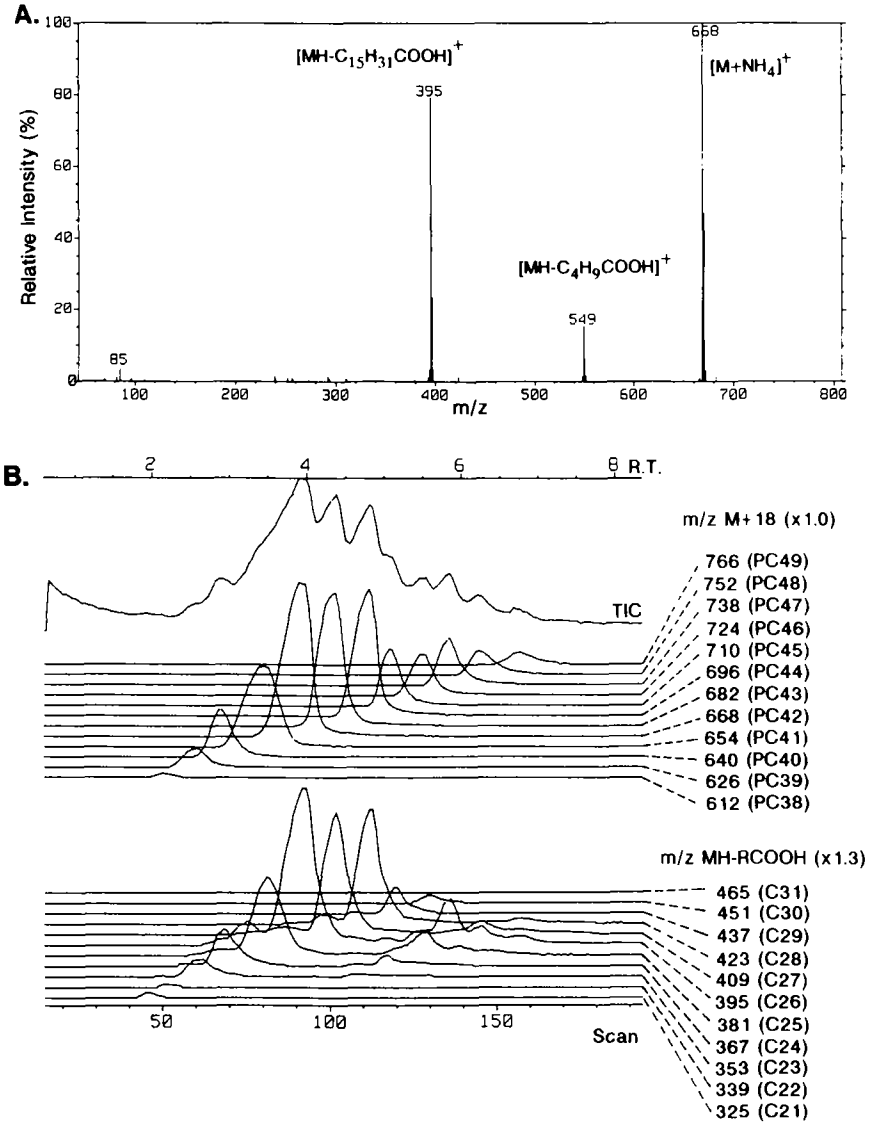

FIG. 4. Ammonia chemical ionization GC–MS for intact diesters IIb. (A) Mass spectra for the largest peak of diesters IIb. (B) Mass chromatograms monitoring the $[M+NH_4]^+$ and $[MH-RCOOH]^+$ ions. (R = C_nH_{2n+1}, n ⩾ 13)

species deduced from the monitoring of the $[M+18]^+$ and $[MH-RCOOH]^+$ ions, shown in Figure 4B.

DISCUSSION

Wax diesters are widely distributed in the surface lipids from a variety of animals (2). They are of two types: diesters of hydroxy fatty acids with fatty acids and fatty alcohols (Type I), and diesters of alkanediols with fatty acids (Type II) (4). For primates, Type II diesters were found, by TLC, in the surface lipids of the baboon, and the surface lipids from the chimpanzee contained only sterol esters (2).

The present study showed that the major constituents of the monkey skin-surface lipids were cholesteryl esters, cholesterol and two species of Type II alkanediol diesters. Type IIa migrated like Type II diesters from the rat on TLC, and contained 1,2-alkanediols esterified with two equivalents of long-chain fatty acids; the shorter-chain ($<C_{20}$) fatty acids predominated at position 1 and the longer-chain ($\geqslant C_{20}$) fatty acids predominated at position 2. This distribution pattern for the fatty acids was similar to that for the hamster diol diesters (17), but the 1,2-alkanediols from the hamster diesters, with its major constituent being 15-methyl-1,2-hexadecanediol (17), had shorter chains than those from the monkey. On the contrary, in the mouse diol diesters, although the chain lengths of the constituent 1,2-alkanediols were close to those of the monkey, the shorter-chain acids were found exclusively at position 2 (17). Consequently, although Type II diesters have been found widely in a variety of animals, including the rat (4), mouse (2), guinea pig (5) and hamster (17), there is a pronounced difference between species with regard to the composition of the fatty acids and diols, and in the distribution pattern for the fatty acids.

The other diesters (IIb) contained 1,2-alkanediols esterified with one equivalent of the short-chain (C_4-C_5)

fatty acid at position 1 and one equivalent of the long-chain (C_{14}-C_{26}) acid at position 2. Type II diesters containing one equimolar of isovaleric acid were found in the dog surface lipids. This structure has been assumed on the basis of the quantitative GC analysis of the constituent short- and long-chain fatty acids (6). In the present study, CI-GC-MS analysis of the intact diesters IIb from the monkey surface lipids presented unambiguous evidence supporting the proposed structure. Furthermore, it was shown that CI-GC-MS was useful for the molecular species analysis of diesters IIb.

The unsaturated fatty acids in the human skin-surface lipids had a characteristic feature (10). They contained straight- and branched-chain components having n-10 double bonds, produced by Δ^6-desaturation, that normally does not occur in other animal tissues (10). In contrast, the unsaturated fatty acids from the monkey surface lipids, that were contained only in 1.5–5.4%, were exclusively straight-chain components having n-7 or n-9 double bonds and having a wide range of chain lengths (C_{18}–C_{34}). The monkey sebaceous gland probably did not have the fatty acid desaturase system. It seems probable that the unsaturated fatty acids originated exclusively from the surrounding tissues and were incorporated into the surface lipids directly or after undergoing chain elongation.

Most animals, except for a few species (2,11), have branched-chain fatty acids as a major constituent of the surface lipids, but information regarding the position of the methyl branching has not been fully provided. The present study showed that the monkey surface lipids contained the monobranched-chain fatty acids having iso and anteiso structures. In general, the iso and anteiso fatty acids of odd carbon number, and iso acids of even carbon number are synthesized by fatty acid synthetase (FAS) from the primer of isovaleryl-CoA, 2-methylbutyryl-CoA and isobutyryl-CoA, respectively (10). In contrast to the human surface lipids (19), the monkey lipids did not contain the branched-chain acids with branches located at the even numbered carbon atoms, except the terminal. These acids, having internal methyl branches, are synthesized with methylmalonyl-CoA as a C_2 unit (20). The present study indicates the diversity in the substrate specificity of the FAS in sebaceous glands among species.

The branched-chain fatty acid in both diesters IIa and IIb shows a two-cluster profile, with maxima around C_{16}-C_{17} and C_{21}-C_{22}. Unsaturated fatty acids from diesters IIa show a three-cluster profile, with maxima at C_{18}, C_{22} and C_{33}. It is probable that C_{16}-C_{17} fatty acids, synthesized by FAS, were elongated by one or two chain-elongation systems, as in the bovine meibomian gland (21).

The 1,2-alkanediols in the monkey surface lipids were saturated components having straight- and branched-chain (iso and anteiso) structures. The diversity in the 1,2-alkanediols has been observed among a variety of animals (2,4–6,8,11). Kolattukudy et al. (22) indicated that the 1,2-alkanediols in the sparrow uropygial gland were derived from 2-hydroxy fatty acids by enzymatic reduction. However, the monkey surface lipids did not contain 2-hydroxy fatty acids. Similarly, the dog and mouse surface lipids had Type II diesters, but not Type I diesters containing 2-hydroxy fatty acids, whereas the rat surface lipids had both Types I and II diesters (2). In the former group, synthesized 2-hydroxy fatty acids might be reduced rapidly to the corresponding 1,2-diols.

In addition to isovaleric acid that has been reported as a short-chain component of the dog diesters (6), the current study showed that 2-methylbutyric acid and isobutyric acid were present in the diester IIb in the monkey. This result suggests that these short-chain fatty acids, that are assumed to be abundantly present as primers of fatty acid synthesis in the sebaceous glands, can be incorporated into the diester IIb, but that the enzyme responsible for the incorporation of these short-chain fatty acids has a relatively high specificity for isovaleric acid.

The sterol fraction from the sterol esters, as well as from the free sterols, contained solely cholesterol. The intermediate of cholesterol biosynthesis, such as lathosterol, that was found in the surface lipids of the guinea pig (5) and dog (6), was not detected in the monkey surface lipids.

These results provide detailed evidence regarding the diversity in skin-surface lipids among species, and the occurrence of the constituents.

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REFERENCES

1. Wolff, M.S. (1984) *Anal. Chem. 56,* 1492–1496.
2. Nicholaides, N., Fu, H.C., and Ansari, M.N.A. (1970) *Lipids 5,* 299–307.
3. Lindholm, J.S., McCormick, J.M., Colton VI, S.W., and Downing, D.T. (1981) *Comp. Biochem. Physiol. 69B,* 75–78.
4. Nikkari, T., and Haahti, E. (1968) *Biochim. Biophys. Acta 164,* 294–305.
5. Downing, D.T., and Sharaf, D.M. (1976) *Biochim. Biophys. Acta 431,* 378–389.
6. Sharaf, D.M., Clark, S.J., and Downing, D.T. (1977) *Lipids 12,* 786–790.
7. Downing, D.T., and Colton VI, S.W. (1980) *Lipids 13,* 323–327.
8. Yeung, D., Nacht, S., and Cover, R.E. (1981) *Biochim. Biophys. Acta 663,* 524–535.
9. Nicholaides, N., Fu, H.C., and Rice, G.R. (1968) *J. Invest. Dermatol. 51,* 83–89.
10. Nicholaides, N. (1974) *Science 186,* 19–26.
11. Downing, D.T., and Lindholm, J.S. (1982) *Comp. Biochem. Physiol. 73B,* 327–330.
12. Stewart, M.E., and Downing, D.T. (1981) *Lipids 16,* 355–359.
13. Lemieux, R.U., and von Rudloff, E. (1955) *Can. J. Chem. 33,* 1701–1709.
14. Apon, J.M.B., and Nicholaides, N. (1975) *J. Chromatogr. Sci. 13,* 467–473.
15. Andersson, B.A., and Holman, R.T. (1975) *Lipids 10,* 716–718.
16. Miwa, H., and Yamamoto, M. (1986) *J. Chromatogr. 351,* 275–282.
17. Schmid, P.C., and Schmid, H.H.O. (1978) *J. Lipid Res. 19,* 894–898.
18. Miwa, H., and Yamamoto, M. (1987) *J. Chromatogr. 416,* 237–245.
19. Nicholaides, N., and Apon, J.M.B. (1977) *Biomed. Mass Spectrom. 4,* 337–347.
20. Seyama, Y., and Kawaguchi, A. (1987) in *Pyridine Nucleotide Coenzymes: Chemical, Biochemical, and Medical Aspects* (Dolphin, D., Poulson, R., and Avramovic, O., eds.) Vol. 2B, pp. 381–431, John Wiley & Sons, Inc., New York, NY.
21. Anderson, G.J., and Kolattukudy, P.E. (1985) *Arch. Biochem. Biophys. 237,* 177–185.
22. Kolattukudy, P.E. (1972) *Biochem. Biophys. Res. Commun. 49,* 1376–1383.

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Integral Lipids of Human Hair

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It has long been recognized that hair is coated with nonpolar lipids originating in the sebaceous glands, and recently it has been shown that hair also contains cholesterol sulfate and small amounts of ceramides, similar to those found in the keratinized portion of the epidermis. In the present study, it is demonstrated that significant amounts of several additional lipids are tightly associated with hair in such a way as to be highly resistant to solvent extraction.

These integral hair lipids included cholesterol sulfate (3.3 mg/g of extracted hair), cholesterol (0.6 mg/g), fatty alcohols (0.2 mg/g) and free fatty acids (4.3 mg/g). The principal fatty acid, comprising 40% of the total fatty acids, was identified as 18-methyl-eicosanoic acid by cochromatography with authentic standard on gas-liquid chromatography (GLC) and by mass spectrometry (MS).

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Human scalp hairs consist of a central medulla of loosely connected keratinized cells surrounded by more-tightly-packed, 80–100 μ-long cortical cells with 6 to 10 outer layers of flattened, rectangular cuticle cells (1). Late in the sequence of differentiative events leading to formation of the mature cuticle and cortex cells, a multilayered material is deposited between the bounding membranes of the individual cells to form membrane complexes (2–4). Although it is chemically undefined, the intercellular material (3,4) has been referred to as a cement substance (3). The chemical nature of the cell periphery in hair, like that of the cornified epidermal cell, also has been somewhat of a puzzle because hair does not contain the usual membrane-forming phospholipids (5).

Associated with the hair follicles are sebaceous glands that provide abundant nonpolar lipids to the skin surface and the hair (6,7). While these sebaceous lipids may serve to lubricate and waterproof, they are not membrane-forming lipids. Recently, it has been demonstrated that hair from several species of mammals contains cholesterol sulfate and several series of ceramides similar to those found in the keratinized portion of the epidermis, or stratum corneum, and it has been postulated that these lipids are of structural significance in hair (5). However, the amounts of these polar lipids were low, and it was uncertain as to whether they provided enough material to account for the observed membranes.

More recently, the occurrence of significant amounts of covalently bound lipid in the stratum corneum has been demonstrated (8). This material consists mainly of an unusual ω-hydroxyacylsphingosine ester-linked to a cross-linked protein envelope to form what appears like a plasma membrane (8,9). It is actually an integral part of the cell envelope and can be removed from the cell surface only after saponification (8,9).

The purpose of the present investigation was to determine whether human hair, a keratinized epidermal appendage, contains covalently bound lipids as found in the stratum corneum. To answer this question, one pooled sample of hair clippings from several people and samples from three individuals were obtained. After extensive extraction with chloroform/methanol mixtures to remove all of the extractable lipids, the hair samples were saponified and reextracted. Additional lipids recovered in this way included major amounts of cholesterol sulfate (3.3 mg/g extracted hair) and free fatty acids (4.3 mg/g) and small amounts of cholesterol, fatty alcohol and several unidentified components. The major fatty acid, comprising 40% of the total fatty acids, proved to be 18-methyl-eicosanoic acid.

MATERIALS AND METHODS

Preparation of delipidized hair. Samples of human hair were repeatedly rinsed with distilled water. They were then extracted, first with methanol and then with chloroform/methanol 2:1, 1:1 and 1:2, for 2 hr each, at room temperature. Each of the chloroform/methanol extractions was then repeated for 24 hr. Finally, the hair was extracted with chloroform in a Soxhlet extractor, continuously for 24 hr. The extracted hair was thoroughly dried en vacuo and weighed. One large sample (100 g) of pooled hair from several individuals and smaller (3.5–8.9 g) hair samples from three individuals were processed by this method.

Saponification and recovery of constitutive lipids. Extracted hair samples (3–5 g after extraction) were saponified by heating for 2 hr at 60 C in 200 ml 1 M NaOH in 90% methanol. The reaction was cooled to room temperature, and 88 ml of water and 360 ml of chloroform were added. The mixture was shaken and the phases were separated in a separatory funnel. The lower chloroform layer was transferred to a flask, and the upper phase and hair residue were acidified by addition of 50 ml of 6 N HCl. The acidified upper phase was extracted with an additional 360 ml of chloroform, and the combined chloroform extracts were taken to dryness with a rotary evaporator. The dried residue was dissolved in 5 ml chloroform:methanol, 2:1, and washed with 1 ml of 2 M KCl containing 0.1 M HCl. The lower chloroform phase then was filtered through a solvent-resistant, 0.5-micron millipore filter, and the filtrate was collected and taken to dryness in a tared glass tube. The wt of the dried lipid was determined, and chloroform:methanol was added to produce a solution containing 20 mg lipid per ml.

Thin-layer chromatography (TLC). Analytical TLC was done on 0.25-mm layers of Silica Gel G (E.M. Reagents, Darmstadt, West Germany) on 20 × 20 cm glass plates. Samples were applied 2 cm from the bottom

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Abbreviations: ECL, equivalent chain lengths; GLC, gas-liquid chromatography; MS, mass spectrometry; TLC, thin-layer chromatography.

edge of the plate, which was developed first to 9 cm with chloroform/methanol/acetic acid/water (40:10:1:1), and then to 20 cm with hexane/ether/acetic acid (70:30:1). After drying, the plate was sprayed with 50% sulfuric acid and charred by slowly heating to 220 C. The charred chromatograms were quantitated by photodensitometry (10) with a Shimadzu model CS-930 photodensitometer. Known amounts of stearic acid, stearyl alcohol, cholesterol and cholesterol sulfate were used as standards.

Fatty acids, fatty alcohols and the sterol fraction were isolated by preparative TLC on 0.5-mm Silica Gel H (E.M. Reagents) using a mobile phase of hexane/ether/acetic acid (70:30:1). Cholesterol sulfate was isolated using chloroform/methanol/acetic acid/water (40:10:1:1) as the mobile phase.

The isolated fractions, as well as the solvolysis product derived from cholesterol sulfate, were checked for purity by TLC with solvent systems of hexane/ether/acetic acid (70:30:1), chloroform/methanol/acetic acid (190:9:1), chloroform/methanol/water (40:10:1) and chloroform/methanol/acetic acid/water (40:10:1:1). Each fraction was judged to be chromatographically pure.

Acetylated sterols, acetylated fatty alcohols and fatty acid methyl esters were purified by preparative TLC using a mobile phase of toluene.

Chemical procedures. The isolated fatty acid fraction was converted to methyl esters by treatment with 10% BCl_3 in methanol at 50 C for 1 hr. The reaction mixture was dried under nitrogen.

Cholesterol sulfate was solvolyzed overnight by treatment with 10% BCl_3 in methanol at 55 C. The sample was dried under nitrogen.

The free sterol, fatty alcohol and the sterol obtained after solvolysis of cholesterol sulfate were acetylated by treatment with acetic anhydride in pyridine at room temperature for 2 hr. Excess reagents were removed by evaporation under a gentle stream of nitrogen.

Gas-liquid chromatography (GLC). The fatty acid methyl esters were examined by GLC on a 50 m CP-SIL 88 quartz capillary column (Chrompack, Inc., Bridgewater, NJ). An initial temperature of 160 C was maintained for 5 min, after which the temperature was increased linearly at a rate of 5 C/min, until 220 C was reached. The final temperature was maintained until no further peaks eluted. The fatty acid methyl esters also were examined isothermally at 160 C and 175 C on the CP-SIL 88 column, as well as at 200 C on a nonpolar 25-m BP-1 quartz capillary column (Scientific Glass Engineering, Inc., Austin, TX).

Standard fatty acid methyl ester mixtures included 16:0, 18:0, 18:1, 18:2, 18:3, 20:0 (15A, NuChek Prep, Elysian, MN); 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 (CE1-62, NuChek Prep); 20:0, 20:1, 20:4, 22:1, 22:6 (L-209, Applied Science, State College, PA); and fatty acids isolated from wool wax (11).

GLC of fractions isolated by argentation-TLC was used to confirm the identification of saturated and monoenoic fatty acid methyl esters.

The acetylated sterols derived from both the free sterol fraction and the sterol sulfate fraction were examined by GLC with the CP-SIL 88 column at 220 C and on BP-1 at 290 C. Authentic cholesterol acetate and cholestanol acetate were used as standards.

The fatty alcohol acetates also were examined by GLC with the CP-SIL 88 column, both isothermally at 160 C and with the temperature program described above. Standard fatty alcohol acetates included C14:0, C16:0, C18:0 and C20:0.

Mass spectrometry. Fatty acid methyl esters were separated on a 25-m CP-SIL 5 quartz capillary column operated with a temperature program, starting at 150 C and increasing 5 C/min, until a final temperature of 250 C was attained. Mass spectra were recorded with a Nermag R 10-10 C mass spectrometer with an ionizing voltage of 70 eV. A range of mass/charge from 40 through 600 was examined.

RESULTS

Composition of constitutive hair lipids. A typical densitometer tracing of a thin-layer chromatogram of the lipids released by saponification of previously extracted hair is presented as Figure 1. As described below, the most prominent components were identified as free fatty acids and cholesterol sulfate, and minor components included cholesterol and fatty alcohols. Some lipid material appearing between R_f 0.2–0.4 was not identified and variable amounts of origin material not thought to be lipid were sometimes present. The quantitative results of such TLC analyses are summarized in Table 1.

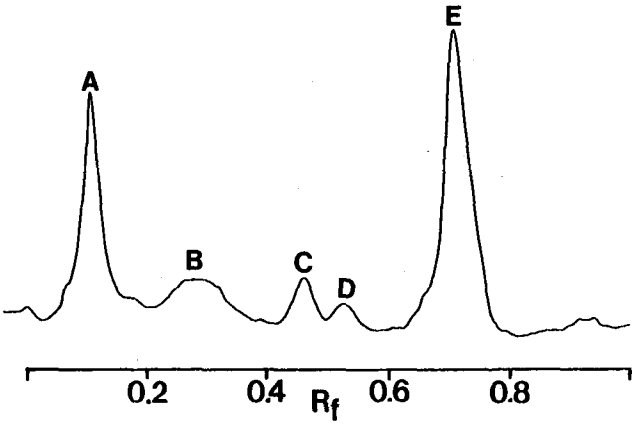

FIG. 1. Densitometer tracing of a thin-layer chromatogram of the constitutive lipids of hair. The chromatogram was developed to R_f 0.30 with chloroform/methanol/water/acetic acid (40:10:1:1) and then to the top with hexane/ether/acetic acid (70:30:1). The charred chromatogram was scanned with a Shimadzu model CS-930 photodensitometer. Peak identification: A, cholesterol sulfate; B, unidentified; C, cholesterol; D, fatty alcohols; E, fatty acids.

TABLE 1

Constitutive Lipids of Human Hair

	Mg lipid per g extracted hair			
Sample	Fatty acid	Cholesterol sulfate	Cholesterol	Fatty alcohol
A	4.7	3.8	0.7	0.1
B	4.2	3.2	0.4	0.2
C	3.2	1.8	0.5	0.1
Pool	5.2	4.2	0.6	0.3
Mean	4.3	3.3	0.6	0.2
SD	0.7	0.9	0.1	0.1

Weights of lipid were estimated by quantitative TLC.

Identification of cholesterol sulfate. The identification of cholesterol sulfate was based on its chromatographic and chemical properties. The isolated material behaved like authentic cholesterol sulfate in terms of both its mobility on TLC in several solvent systems and the pink-violet color produced on heating with sulfuric acid prior to charring. Also, the products produced by hydrolysis and hydrolysis plus acetylation of the isolated lipid behaved during TLC and charring like authentic cholesterol and cholesterol acetate respectively. Finally, the acetylated product gave rise to a single major (96.6% of the total) peak when examined by GLC with either a polar (CP-SIL 88) or nonpolar (BP-1) column. This major component comigrated with authentic cholesterol acetate. Several minor components were not identified.

Identification of free sterol. The isolated free sterol was identified as cholesterol by essentially the same criteria used to identify the sterol released by solvolysis of the sterol sulfate fraction. GLC of the sterol acetate gave one major peak (96.9%) that cochromatographed with cholesterol acetate on both the polar and the nonpolar columns.

Identification of fatty alcohols. The fatty alcohols had the same mobility as authentic fatty alcohols on TLC with mobile phases of hexane/ether/acetic acid (70:30:1) and chloroform/methanol/acetic acid (190:9:1). Like the free sterol, acetylation yielded a single less polar product that migrated on TLC like authentic fatty alcohol acetate.

Examination by GLC revealed a complex mixture. Although the major components accounting for 63.3% of the total were identified as members of the straight-chain saturated series by comparison with standards and construction of plots of carbon number vs log retention time from isothermal chromatograms, the remainder consisted of components with fractional equivalent chain lengths (ECL). The fatty alcohol acetates were identified as either saturated (86%) or unsaturated (14%) after fractionation of the acetates on silver nitrate-impregnated silicic acid. This information is summarized in Table 2.

Identification of fatty acids. The fatty acids were converted to methyl esters and examined by GLC and by GLC-mass spectrometry (MS). A representative chromatogram is shown in Figure 2, and the composition is summarized in Table 3.

The major fatty acid was identified as 18-methyl-eicosanoic acid on the basis of its GLC behavior and its mass spectrum, presented as Figure 3. ECL values of 20.74 and 20.67 were determined from isothermal chromatograms obtained with CP-SIL 88 and BP-1, respectively. A value of 20.72 would be expected for 18-methyl-eicosanoic acid (12). Furthermore, on both the polar and nonpolar columns, the fatty acid methyl ester in question cochromatographed with the C-21 anteiso methyl-branched fatty acid methyl ester derived from wool wax (11).

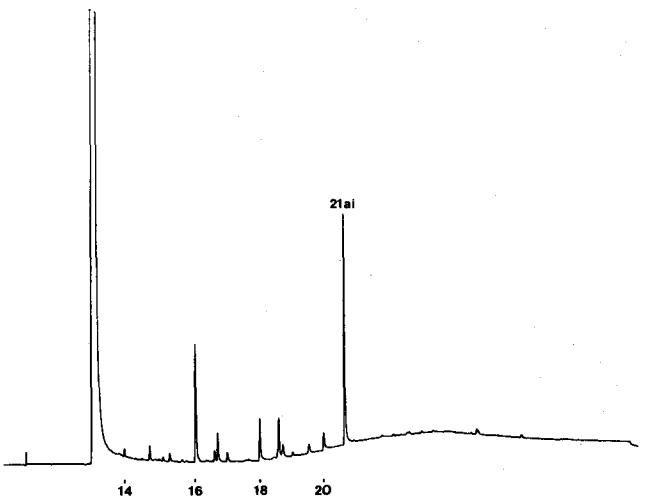

FIG. 2. Gas-liquid chromatogram of methyl esters prepared from the fatty acid fraction of hair constitutive lipids. The methyl esters were chromatographed on a CP-SIL 88 quartz capillary column with a temperature program as explained under Materials and Methods. The straight-chained saturated species and the 21-carbon anteiso-branched component are indicated.

TABLE 2

Composition of Alcohol Acetates

ECL	Chain	%	ECL	Chain	%
12.00	12:0	0.1	20.30	21:0br	0.2
13.00	13:0	0.1	20.38	21:0br	0.2
14.00	14:0	4.7	20.41u	20:1	6.0
15.00	15:0	2.1	20.50u	20:1	1.5
15.53	16:0br	0.3	20.56	21:0br	1.4
16.00	16:0	10.6	20.71	21:0br	4.0
16.30	17:0br	0.2	21.00	21:0	1.8
16.40	17:0br	0.1	21.08	22:0br	0.3
16.53	17:0br	0.5	21.43	22:0br	0.2
16.70	17:0br	2.6	21.57	22:0br	2.5
17.00	17:0	1.1	22.00	22:0	5.2
17.53u	17:1	0.6	22.28u	22:1	2.7
18.00	18:0	17.0	22.33u	22:1	2.6
18.40u	18:1	0.4	22.43	23:0br	0.7
18.50u	18:1	0.2	22.49	23:0br	0.2
18.54	19:0br	0.9	22.59	23:0br	0.4
18.71	19:0br	1.2	22.72	23:0br	1.8
19.00	19:0	2.0	23.00	23:0	1.7
19.49	20:0br	0.1	23.36	24:0br	0.7
19.55	20:0br	2.3	23.59	24:0br	1.8
20.00	20:0	11.6	24.00	24:0	5.3

Monounsaturated species, are designated by a u following the ECL value, as well as by the N:1 designation of the chain structure. It is not known if any of the unsaturated chains also are branched. For the saturated species, branching is indicated by a br at the end of the chain assignment.

TABLE 3

Composition of Covalently Bound Fatty Acids

Chain	Wt %
14:0	0.8
15:0	1.1
16:0	18.3
16:1	1.9
17:0br	5.5
17:0	1.9
18:0	7.0
18:1	3.9
18:0br	8.4
20:0	2.3
20:0br	3.5
21:0 anteiso	40.5
others	4.9

Several saturated species indicated by br, appear to be branched.

FIG. 3. Electron impact mass spectrum of methyl-18-methyl-eicosanoate. The molecular ion (M) and other significant fragments are indicated.

The identification was confirmed by the mass spectrum (Fig. 2). The spectrum included a molecular ion at m/e 340, consistent with the formula $C_{22}H_{44}O_2$. The spectrum also included a number of features characteristic of fatty acid methyl esters. These included a base peak at m/e 74, and prominent fragments at m/e 87 and 143. Also, characteristic chain-degradation fragments (C3–C8) were present. Of particular significance are the peaks at M-29 and M-31 (13). M-31 is present in all methyl esters and is thought to represent formation of an acylium ion. The M-29 represents loss of ethyl, and is more prominent than M-31 only in cases of anteiso methyl branching. In the present spectra, M-29/M-31 = 2.2.

DISCUSSION

The present results demonstrate that human scalp hair contains constitutive lipids that are not removable by exhaustive extraction with chloroform/methanol mixtures. These lipids, that together represent 0.7–1.3% of the weight of hair, were recovered only after treatment with alkaline methanol. The major lipid class recovered in this way is fatty acid, which is present at a level of 4.3 mg per g of hair. This fatty acid is likely attached to the cell surface through ester or thioester linkages. Cholesterol sulfate is the second most abundant of the hair constitutive lipids and constitutes 3.3 mg of each g of hair. The nature of the strong interaction of this lipid with hair is uncertain; however, it may be present in the form of an insoluble salt rather than a covalent adduct. Cholesterol and fatty alcohol, both relatively minor constitutive lipids, could be esterified to acidic groups at the cell surface.

Recently, covalently bound lipids have been identified in mammalian epidermis (8,9,14). In contrast to the present findings, the major bound lipid found in the epidermis is an unusual ceramide consisting of a long-chain (30–34 carbon) ω-hydroxy acid in amide linkage with sphingosine. This is the predominant bound lipid in the stratum corneum, where it is thought to comprise a lipid envelope on the exterior of each keratinized cell. By analogy, the constitutive lipids of hair also may be bound to the cell surface to make a pliable but environmentally resistant lipid coat or envelope.

Perhaps the most striking result of the present investigation was the finding that 40% of the fatty acid bound to hair is 18-methyl-eicosanoic acid. This anteiso methyl-branched fatty acid is present in small amounts in human sebum and vernix caseosa (15) and in wool wax (11), but is not abundant in any source other than hair. Shorter 13–17 carbon anteiso fatty acids are major lipid components of some microorganisms (16–18), where it is thought that they are used, instead of unsaturated fatty acids, to make fluid membranes. It seems feasible that the 21-carbon anteiso fatty acid in hair also may serve to fluidize membranes. In this capacity, the branched-chain acid would have an advantage over unsaturated fatty acids in being resistant to oxidative damage on long exposure to the atmosphere.

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REFERENCES

1. Chapman, R.E. (1986) in *Biology of the Integument*, (Bereiter-Hahn, J., Matoltsy, A.G., and Richards, K.S., eds.) pp. 293–311, Springer-Verlag, Berlin, West Germany.
2. Birbeck, M.S.C., and Mercer, E.H. (1956) *Nature 178*, 985–986.
3. Rogers, G.E. (1959) *Ann. N.Y. Acad. Sci. 83*, 378–399.
4. Parakkal, P.F., and Matoltsy, A.G. (1964) *J. Invest. Dermatol. 43*, 23–34.
5. Wix, M.A., Wertz, P.W., and Downing, D.T. (1987) *Comp. Biochem. Physiol. 86B*, 671–673.
6. Nicolaides, N., Fu, H.C., and Rice, G.R. (1968) *J. Invest. Dermatol. 51*, 83–89.
7. Stewart, M.E., Downing, D.T., and Strauss, J.S. (1983) *Dermatol. Clin. 1*, 335–344.
8. Wertz, P.W., and Downing, D.T. (1987) *Biochim. Biophys. Acta 917*, 108–111.
9. Swartzendruber, D.S., Wertz, P.W., Madison, K.C., and Downing, D.T. (1987) *J. Invest. Dermatol. 88*, 709–713.
10. Downing, D.T. (1968) *J. Chromatogr. 38*, 91–99.
11. Downing, D.T., Kranz, F.H., and Murray, K.E. (1960) *Aust. J. Chem. 13*, 80–94.
12. Nicolaides, N., and Apon, J.M.B. (1976) *Lipids 11*, 781–790.
13. Abrahamsson, S., Stallberg-Stenhagen, S., and Stenhagen, E. (1963) *Prog. Chem. Fats Other Lipids 7*, 1–164.
14. Wertz, P.W., and Downing, D.T. (1986) *Biochem. Biophys. Res. Commun. 137*, 992–997.
15. Nicolaides, N., Fu, H.C., Ansari, M.N.A., and Rice, G.R. (1972) *Lipids 7*, 506–517.
16. Zuneda, M.C., Guillenea, J.J., Dominguez, J.B., Prado, A., and Goni, F.M. (1984) *Lipids 19*, 223–228.
17. Song, Y., Sawa, T., Tsuvhiya, M., Kondo, S., Hamada, M., and Umezawa, H. (1981) *J. Antibiot. Tokyo 34*, 980–983.
18. Kaneda, T., and Smith, E.J. (1980) *Can. J. Microbiol. 26*, 893–898.

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Fatty Acid Metabolism in Renal Ischemia

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The increase in free fatty acids in the ischemic tissue is a consistent observation and these free fatty acids are considered to play a role in the cellular toxicity. To elucidate the cause of higher levels of free fatty acids in ischemic tissue, we examined the catabolism of fatty acids. The β-oxidation of lignoceric (24:0), palmitic (16:0) and octanoic (8:0) acids and the peroxidation of fatty acids were measured at different times of renal ischemia in whole kidney homogenate. The enzymatic activities for the oxidation of fatty acids decreased with the increase in ischemia time. However, the lipid peroxide levels increased 2.5-fold of control with ischemic injury. Sixty min of ischemia reduced the rate of oxidation of octanoic, palmitic and lignoceric acids by 57, 59 and 69%, respectively. Almost similar loss of fatty acid oxidation activity was observed in the peroxisomes and mitochondria. These data suggest that loss of mitochondrial and peroxisomal fatty acid β-oxidation enzyme activities from ischemic injury may be one of the factors responsible for the higher levels of free fatty acids. *Lipids 23,* 882–884 (1988)

Renal ischemia is of clinical interest because of its role in acute renal failure (1). Following ischemia, changes that occur in the membranes and the toxic effect of metabolic products lead to the activation of calcium-dependent catabolic enzymes (2). There are numerous intracellular events taking place during the initial ischemic period, such as influx and efflux of ions, nuclear chromatin denaturation, swelling or shrinking of intracellular compartments and changes in lipid metabolism. These injurious events lead to irreversible cell injury and cell death within a relatively short period of time (3). Sixty min of ischemia in the rat kidney produces irreversible ischemic damage correlated with excessive degradation of phosphatidylcholine and an increase in free fatty acids, lysophosphatidylcholine and diacylglycerol (4). These changes in membrane lipids and/or the fatty acid toxicity may result in alterations in the membrane enzyme activities that may, in turn, lead to loss of organelle functions and cell damage. This study reports that ischemic injury leads to loss of the fatty acid β-oxidation activities of both the mitochondria and peroxisomes.

MATERIALS AND METHODS

Materials. [1-^{14}C]Palmitic acid (58.7 mCi/mmol) was purchased from New England Nuclear (Boston, MA). [1-^{14}C]Lignoceric acid (53.8 mCi/mmol) was synthesized in our laboratory (5). Malate, FAD, NAD, L-carnitine and α-cyclodextrin were purchased from Sigma Chemical Co. (St. Louis, MO). ATP and CoASH were obtained from PL Biochemicals (Milwaukee, WI). Thiobarbituric acid, sodium dodecyl sulfate and tetraethoxypropane were products of Sigma Chemical Co. (St. Louis, MO).

Operative procedure. Male Sprague-Dawley rats, weighing 250 g, were obtained from the Charles River Breeding Laboratories (Wilmington, MA). The rats were anesthetized with sodium pentobarbital (50 mg/kg/rat). Heparin (250 units) was injected into the left femoral vein to prevent coagulation. Under clean, but not sterile conditions, the abdomen was opened through a midline incision. The left kidney was carefully mobilized and the renal vessels were ligated with 40 silk suture. The abdomen was closed with running suture. At 15, 30, 60, 90, 120 and 180 min, the abdomen was reopened, both kidneys were removed and immediately placed in ice-cold 0.25 M sucrose in 10 mM MOPS (3-(N-morpholino)propane sulfonic acid) buffer, pH 7.4. The kidneys were individually homogenized in 20 ml of the same solution using a glass-teflon homogenizer. The mitochondria from control and ischemic kidney were 85–90% intact as determined by glutamate dehydrogenase assay in the presence and absence of Triton X-100 (6). Fractions of whole kidney homogenate were used for different assays. The right kidney served as the nonischemic control in all cases.

Fatty acid oxidation to acetate. Enzyme activity for the oxidation of [1-^{14}C]labeled fatty acids to acetate (water soluble products) was measured with modification of the previous procedure (7). The reaction mixture (0.5 ml) contained 12 μM [1-^{14}C]labeled fatty acid coated in Celite, 20 mM MOPS buffer (pH 7.8), 30 mM KCl, 8 mM ATP, 0.25 mM NAD, 0.17 mM FAD, 2.5 mM L-carnitine, 0.08 mM CoASH and 1 mg α-cyclodextrin. The reaction mixture was incubated in a shaking water bath at 37 C and started by addition of 100 μl of whole kidney homogenate (500–800 μg of protein). It was stopped 30 min later with 1.25 ml of 1 M potassium hydroxide. The supernatant fraction was transferred to another tube, incubated at 60 C for 1 hr and partitioned by the procedure of Folch et al. (8), after neutralization with acid. The radioactivity in the upper layer was measured and the amount of radioactivity is an index of the [1-^{14}C]labeled fatty acid oxidized to acetate. Protein was measured by the procedure of Lowry et al. (9), using bovine serum albumin as standard.

Lipid peroxidation. Lipid peroxidation was measured using a slight modification of the thiobarbituric acid (TBA) method (10). The reaction mixture contained 0.25 ml of whole kidney homogenate (1,250–2,000 μg of protein), 0.2 ml of 8.1% sodium dodecyl sulfate, 1 ml of 20% acetic acid (pH 3.5) and 1 ml of 0.8% TBA in water. The mixture was heated at 95 C in an oil bath for 60 min. After cooling in ice-cold water, 2.5 ml of the mixture of n-butanol and pyridine (15:1) were added, vortexed and centrifuged at 4,000 rpm for 10 min. The absorbance of organic layer (upper layer) was measured at 532 nm. The standard used was tetraethoxypropane, as modified by Shlafer and Shepard (11).

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Abbreviations: ATP, adenosine triphosphate; CoASH, coenzyme A; FAD, flavin adenine dinucleotide; MOPS, 3-(N-morpholino) propane sulfonic acid; NAD, nicotinamide adenine dinucleotide; TBA, thiobarbituric acid.

RESULTS AND DISCUSSION

Following ischemia, membrane changes appear to play a crucial role determining integrity of the ischemic cell. The accelerated catabolism of phospholipids by the abnormal increase in the activities of intracellular calcium-activated phospholipases in ischemia is associated with membrane dysfunction (12–13). Increase in free fatty acids and decrease in the level of ATP are consistent findings in ischemic injury. We examined the effect of ischemia on the fatty acid catabolism in the kidney.

Fatty acids can be catabolized by α-, β- or ω-oxidation, but β-oxidation is the predominant pathway. At the subcellular level, fatty acids can be β-oxidized in mitochondria and peroxisomes (14–17). Although both β-oxidation systems are functionally similar with respect to degradation of fatty acids by two carbon units per cycle, they are structurally different (16). Predominant oxidation of short-chain ($< C_{10}$) fatty acids is in mitochondria (15) and of very-long-chain fatty acids (lignoceric acid, C_{24}) is in peroxisomes (18,19), whereas long-chain (C_{12}–C_{20}) fatty acids are oxidized both in mitochondria and peroxisomes (14–15). The results shown in Figures 1–3 demonstrate that ischemia leads to a decrease in enzyme activities both in mitochondria (Figs. 1 and 2) and peroxisomes (Fig. 3). Sixty min of ischemia reduced the rate of oxidation of octanoic, palmitic and lignoceric acids by 57, 59 and 69%, respectively. Activation of fatty acids to acyl-CoA derivatives is the prerequisite for the oxidation of fatty acids and ATP is needed for this reaction (20–22). The assay system for the measurement of β-oxidation contained 8 mM of ATP for both control and ischemic tissue, thus, decreased activ-

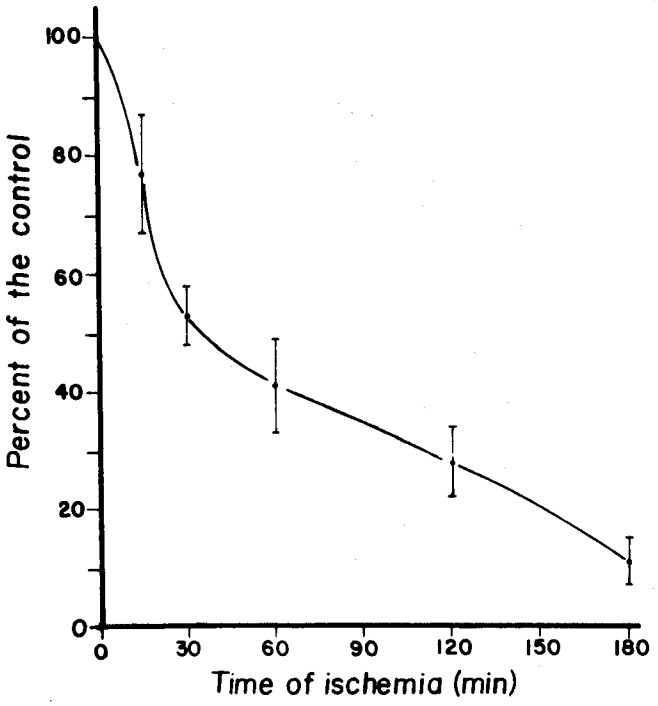

FIG. 2. The rate of β-oxidation of palmitic acid by kidney homogenate with respect to time of ischemia. The enzyme assays were performed in duplicate as described under Methods. The results are expressed as percentage of the control kidney ± SD, (n = 5).

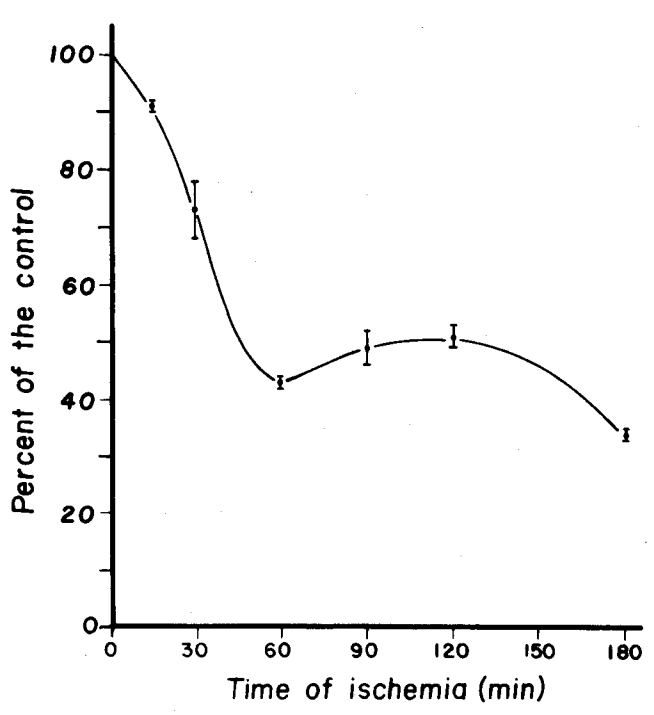

FIG. 1. The rate of β-oxidation of octanoic acid by kidney homogenate with respect to time of ischemia. The enzyme assays were performed in duplicate as described under Methods. The results are expressed as percentage of the control kidneys ± SD (n = 5).

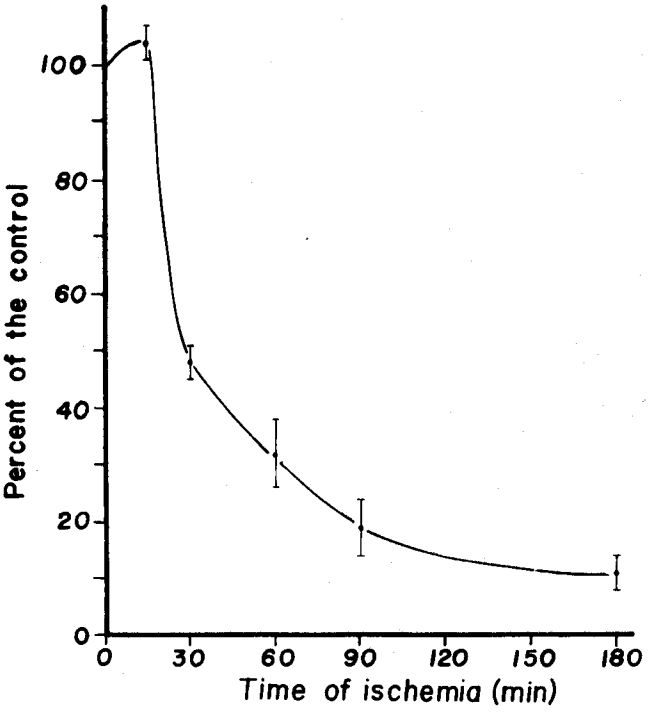

FIG. 3. The rate of β-oxidation of lignoceric acid by kidney homogenate with respect to time of ischemia. The enzyme assays were performed in duplicate as described under Methods. The results are expressed as percentage of the control kidneys ± SD (n = 5).

ity of β-oxidation is not due to the limited amount of ATP present in the ischemic tissue. Moreover, the increase in free fatty acids in ischemic tissue (4) was much smaller than exogenously added radiolabel fatty acid to account for any significant dilution of the substrate. Membrane phospholipids contain high amounts of unsaturated fatty acids, especially arachidonic acid (23,24). Degradation of phospholipids by phospholipase A_2 during ischemic injury contributes to higher levels of unsaturated fatty acids (25). The presence of higher levels of unsaturated fatty acids, when their mitochondrial-peroxisomal catabolism is inhibited (Figs. 1-3), may overwhelm the endoplasmic reticulum capacity for their metabolism and result in their auto-oxidation to lipid peroxides (26). In the ischemic kidney, the levels of lipid peroxides were 250% higher than in the control (Fig. 4). The lipid peroxidation is associated with the generation of lipid-derived intermediate products including hydroperoxides, hydroxyaldehydes and malondialdehyde. These intermediates have been suggested to play an important role in cellular damage (27,28). Normally the cell is provided by enzymes with a protective mechanism (superoxide dismutase, catalase and glutathione oxidase) that detoxifies both reactive oxygen metabolites and lipid-derived intermediates. However, in ischemia, this cellular control seems to be disturbed, because levels of lipid peroxides increases with ischemic injury (Fig. 4).

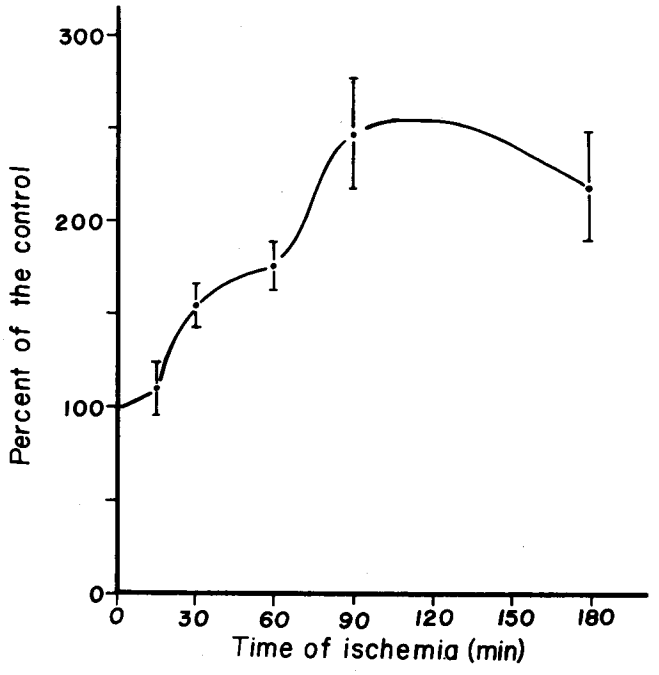

FIG. 4. The levels of lipid peroxides in kidney with respect to time of ischemia. The assays were performed in triplicate as described under Methods. The results are expressed as percentage of the control kidney ± SD, (n = 5).

In summary, the results reported here demonstrate that lipid alterations in membranes and high concentrations of toxic free-fatty acids and lipid peroxides in ischemia may impair the functional properties of subcellular organelles (e.g., mitochondria and peroxisomes).

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REFERENCES

1. Brezis, M., Rosen, S., Silva, P., and Epstein, F.H. (1984) *Kidney Int. 26*, 375-383.
2. White, B.C., Krause, G.S., Aust, S.D., and Eyster, G.E. (1985) *Ann. Emergency Med. 14*, 804-809.
3. Reimer, K.A., and Jennings, R.B. (1971) *Lab. Invest. 25*, 185-195.
4. Matthys, E., Patel, Y., Kreisberg, J., Stewart, J.H., and VenKatachalam, M. (1984) *Kidney Int. 26*, 153-161.
5. Hoshi, M., and Kishimoto, Y. (1973) *J. Biol. Chem. 248*, 4123-4130.
6. Hogeboom, C.A., and Schneider, W.C. (1953) *J. Biol. Chem. 204*, 233-238.
7. Singh, I., Moser, A.E., Goldfischer, S., and Moser, H.W. (1984) *Proc. Natl. Acad. Sci. USA 81*, 4203-4207.
8. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226*, 497-509.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193*, 265-275.
10. Ohkawa, H., Ohishi, N., and Yagi, K. (1979) *Anal. Biochem. 95*, 351-358.
11. Shlafer, M., and Shepard, B.M. (1984) *Anal. Biochem. 137*, 269-276.
12. Chien, K.R., Abrams, J., Serroni, A., Martin, J.T., and Farber, J.L. (1978). *J. Biol. Chem. 253*, 4809-4817.
13. Chien, K.R., Pfau, R.G., and Farber, J.L. (1979) *Am. J. Pathol. 97*, 505-529.
14. Lazarow, P.B., and De Duve, C. (1976) *Proc. Natl. Acad. Sci. USA 73*, 2043-2046.
15. Mannaerts, G.P., and Debeer, L.J. (1982) *Ann. N.Y. Acad. Sci. 386*, 30-38.
16. Hashimoto, T. (1982) *Ann. N.Y. Acad. Sci. 386*, 5-12.
17. Osmundsen, H. (1982) *Ann. N.Y. Acad. Sci. 386*, 13-27.
18. Singh, H., and Poulos, A.A. (1986) *Arch. Biochem. Biophys. 250*, 171-179.
19. Singh R.P., and Singh, I. (1986) *Neurochem. Res. 11*, 281-289.
20. Lipmann, F., Jones, M.E., Black, S., and Flynn, R.M. (1952) *J. Am. Chem. Soc. 74*, 2384-2385.
21. Groot, P.H.E., Scholte, H.R., and Hulsmann, W.C. (1976) *Adv. Lipid. Res. 14*, 75-125.
22. Singh, I., Singh, R.P., Bhushan, A. and Singh, I. (1985) *Arch. Biochem. Biophys. 236*, 418-426.
23. Jordan, R.A., and Schenkman, J.B. (1982) *Biochem. Pharmacol. 31*, 1393-1400.
24. Rouser, G., Nelson, G.J., Fleischer, S., and Simon, G. (1968) In *Biological Membranes - Physical Fact and Function*, (Chapman, D., ed.) Vol I, pp. 5-69, Academic Press, New York, NY.
25. Smith, M.W., Collan, Y., Kahng, M.W., and Trump, B.F. (1980) *Biochim. Biophys. Acta 618*, 192-201.
26. Porter, N.A. (1986) *Acc. Chem. Res. 19*, 262-268.
27. Tappel, A.L. (1973) *Fed. Proc. 32*, 1870-1874.
28. Lee, F., and Massaro, D. (1979) *Arch. Intern. Med. 139*, 347-350.

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Linoleic Acid-induced Fatty Acid Changes in Platelet and Aorta of the Rat: Effect of Age and Cholesterol

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The influence of age and cholesterol on polyunsaturated fatty acids (PUFA) levels was studied in young and old male Sprague-Dawley rats. Animals were fed a fat-free diet supplemented with 10% (by wt) safflower oil with or without 1% cholesterol for 8 wk. As a result of cholesterol feeding, proportions of linoleic acid (18:2n-6) and dihomo-γ-linolenic acid (20:3n-6) were increased and and that of arachidonic acid (20:4n-6) was decreased in the liver and platelet phospholipids in 64-wk-old rats, suggesting inhibitory effects of cholesterol on 20:4n-6 synthesis from 18:2n-6. The prominent age-dependent effect on the levels of PUFA was a retention of C-22 n-3 PUFA, accompanied by decreased C-22 n-6 PUFA and increased 20:3n-6 in the liver and platelet phospholipids. Ratio of 20:3n-6/20:4n-6 increased in 64-wk-old rats regardless of dietary cholesterol, suggesting depressed Δ5-desaturase with age. In aorta phospholipids, 20:3n-6 content and 20:3n-6/20:4n-6 ratio increased with cholesterol supplementation, but not with age. These results suggest that changes of PUFA composition of platelet phospholipids with age are closely linked with changes in liver phospholipids. The 20:4n-6 content in both platelet and aorta phospholipids is kept constant, despite other n-6 and n-3 PUFA being affected by age.
Lipids 23, 885–890 (1988).

The metabolism of polyunsaturated fatty acids (PUFA) is affected by dietary and metabolic factors (1,2). It is of interest to study PUFA metabolism at different ages because hormonal and metabolic conditions change with age, and because the incidences of diabetes and hypercholesterolemia, which are known to relate to PUFA metabolism (3,4), increase with age (5,6). While an age-dependent decrease of synthesis of desaturated and elongated metabolites of linoleic acid has been shown during the maturation period in rat testicles (7,8), PUFA status in other tissues is less clear. Arachidonic acid (20:4n-6), dihomo-γ-linolenic acid (20:3n-6) and eicosapentaenoic acid (20:5n-3) are the precursors of antiaggregatory prostaglandins I_2, E_1 and I_3, and the potent aggregatory thromboxane A_2 in vascular tissue and platelets (9–13). These PUFA compete with each other for the cyclooxygenase enzyme (12,13). Furthermore, these PUFA also have a lipid-lowering effect (14,15). Therefore, an altered tissue content of these PUFA could contribute to atherogenesis and thrombotic disorders with increasing age. The purpose of the present study was to examine the n-6 and n-3 PUFA profiles in liver, platelet and aorta phospholipids and adipose tissue lipids in weanling and 56-week-old rats, in response to feeding a diet which contained only an n-6 fatty acid (linoleic acid, 18:2n-6) as a PUFA source. The effect of dietary cholesterol, which has been suggested to inhibit Δ6- and Δ5-desaturase activity on PUFA metabolism in immature rats (16), was also examined.

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Abbreviations: GLC, gas liquid chromatography; TLC, thin layer chromatography.

METHODS

Male weanling (3 wk of age) and 56 wk of age Sprague-Dawley rats were obtained from Canadian Hybrid Farms, Halls Harbour, NS. The animals and their dams, for at least several generations, had been fed a nonpurified diet (Purina Rodent Chow) until receipt in our laboratory. After arrival, the rats were randomly divided into four age-matched groups of seven rats each and were fed for eight wk with a 10% (by wt) safflower oil-supplemented fat-free diet that was ca. 16% energy (Teklad Test Diets, Madison, WI). Half of the 14 animals in each different age group also were given 1% (by wt) cholesterol in the diet. The experimental diet composition and the fatty acid composition of Purina Rodent Chow and safflower oil are shown in Tables 1 and 2.

TABLE 1

Composition of the Experimental Diet

	g/kg
Sucrose	607.0
Casein, vitamin-free	193.0
Safflower oil	100.0
Cellulose	50.0
Mineral mix, AIN-76	35.0
Vitamin mix, AIN-76A	10.0
DL-Methionine	3.0
Choline bitartrate	2.0

TABLE 2

Fatty Acid Composition of Purina Rodent Chow and Safflower Oil

Fatty acid	Purina rodent chow	Safflower oil
14:0	2.0	0.1
16:0	20.9	6.6
16:1	3.4	—
18:0	8.8	2.1
18:1	29.9	11.0
18:2n-6	28.5	80.0
18:3n-3	3.0	—
20:4n-6	0.2	—
20:5n-3	1.8	—
22:5n-3	0.4	—
22:6n-3	1.1	—

Results are shown as mg/100 mg total fatty acids present. Total fat in the rodent chow was 45 g/kg.

All animals were kept in groups of two to four in plastic cages and had free access to food and water. One rat was killed because it had been severely wounded by another rat. After the feeding period, cardiac punctures to collect blood were performed under ether anesthesia. Aorta, liver and epididymal fat were removed, trimmed, rinsed in saline, blotted and frozen at −20 C. Macroscopically, no abnormal finding was observed in any of the animals. Platelets were separated from plasma by centrifugation (12), washed by calcium-free Tyrode's buffer and frozen at −20 C. Liver, platelets and aorta lipids were extracted by the method of Bligh and Dyer (17). The phospholipid fractions were separated by thin layer chromatography (TLC), methylated and subjected to gas liquid chromatography (GLC) for determination of fatty acid composition as previously described (18). A 10% Silar 10 C on Gas Chrom Q column was used with a Hewlett-Packard 5880A machine with automated integration. Identification was based on retention time with respect to standard methyl ester mixtures (Nu-Chek Preps, Elysian, MN). As over 95% (by wt) of adipose tissue consists of triglycerides, a small amount of the epididymal fat was directly methylated without extraction.

Total phospholipid concentration in liver lipid extracts was measured by the method of Stewart (19). Liver protein was determined using Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Plasma cholesterol and triglyceride concentrations were determined enzymatically using a Cobas-Bio centrifugal analyzer (Hoffman-La Roche, Nutley, NJ). Statistical analysis was performed using the Tukey-Kramer test preceded by ANOVA (20).

RESULTS

Cholesterol feeding caused a significant increase in the liver wt of both 11-week-old and 64-week-old rats, but had no effect on body wt (Table 3). Liver phospholipid concentration was significantly reduced by cholesterol feeding, but this was not significant when values were expressed as a function of protein concentrations (Table 3). There was no age-dependent difference in liver phospholipid concentration. Cholesterol and triglyceride levels in plasma were higher in the older animals (Table 3). In both young and old animals, feeding cholesterol elevated plasma cholesterol and lowered plasma triglyceride concentrations.

Fatty acid compositions of liver and platelet phospholipids, following administration of safflower oil with or without cholesterol, are shown in Tables 4 and 5. Dietary cholesterol supplementation lead to the following changes: 1) The levels of Δ9-desaturase products (palmitoleic [16:1n-7] or oleic [18:1n-9] acids) are increased; 2) the levels of precursor fatty acids for 20:4n-6 synthesis (18:2n-6, eicosadienoic [20:2n-6] or 20:3n-6 acids) are increased, and in the liver C-22 PUFA (docosatetraenoic [22:4n-6], docosapentaenoic [22:5n-6] acids or docosahexaenoic acid [22:6n-3]) are decreased; and 3) 20:4n-6 is decreased in liver and platelet phospholipids of 64-week-old rats and in platelet, but not in liver, phospholipids of 11-week-old rats.

The effects of age on the fatty acid compositions of the liver and platelet phospholipids are as follows: 1) The levels of 20:3n-6 are increased; 2) the ratios of 20:3n-6/20:4n-6 are increased; 3) the levels of C-22 n-6 PUFA (22:4n-6 or 22:5n-6) are reduced and 20:5n-3, 22:5n-3 or 22:6n-3 are retained; and 4) no apparent influence on the levels of 20:4n-6 in either liver or platelet phospholipids was observed.

The effects of dietary cholesterol on fatty acid compositions in aorta phospholipids were less marked than in liver and platelet phospholipids (Table 6). Increased 20:3n-6 and 20:3n-6/20:4n-6 ratio were the only results of administering cholesterol and were obtained both in 11-week-old and 64-week-old rats. On the other hand, decreased 22:5n-6 and increased 22:5n-3 and 22:6n-3 were observed with aging, regardless of cholesterol supplementation, with 20:3n-6/20:4n-6 ratio being unaffected and 20:4n-6 being kept invariably constant (Table 6).

In adipose tissue lipids, the effects of cholesterol

TABLE 3

Body Weight, Liver Weight and Liver Phospholipid (PL) Concentration in Rats With or Without Cholesterol (CHO) Feeding

	11 Weeks of age		64 Weeks of age	
	CHO(−)	CHO(+)	CHO(−)	CHO(+)
Number	7	7	6	7
Body weight (g)	406 ± 24	443 ± 50	771 ± 94*	788 ± 86*
Liver weight (g)	11.4 ± 0.8	18.9 ± 2.0**	21.5 ± 4.7*	28.2 ± 6.9*,**
Liver PL (μmol/g wet wt)	19.6 ± 1.9	13.4 ± 2.0**	17.3 ± 2.0	13.2 ± 1.0**
Liver PL (μmol/mg protein)	72.3 ± 5.4	62.9 ± 14.5	74.1 ± 10.7	60.9 ± 5.2
Plasma cholesterol (mg/dl)	40 ± 8	65 ± 19	77 ± 18*	91 ± 23*
Plasma triglyceride (mg/dl)	103 ± 31	70 ± 22	265 ± 66*	167 ± 61*

Values are means ± S.D.

*Significantly different than the corresponding young group ($p < 0.05$).

**Significantly different than the age-matched group without cholesterol feeding ($p < 0.05$).

TABLE 4

Fatty Acid Composition of Liver Phospholipids in Rats With or Without Cholesterol (CHO) Feeding

	11 Weeks of age		64 Weeks of age	
	CHO(−)	CHO(+)	CHO(−)	CHO(+)
Number	7	7	6	7
Fatty acid				
16:0	18.4 ± 1.1	19.0 ± 0.7	17.7 ± 1.3	17.7 ± 1.7
16:1n-7	0.4 ± 0.2	1.4 ± 0.2**	1.1 ± 0.6	1.6 ± 0.7
18:0	25.0 ± 6.9	17.1 ± 3.9**	20.1 ± 1.6	16.7 ± 2.6
18:1n-9	3.9 ± 0.3	7.6 ± 1.2**	4.6 ± 2.2	9.3 ± 1.5**
18:2n-6	11.1 ± 1.9	18.5 ± 2.9**	10.3 ± 1.5	17.6 ± 3.8**
18:3n-6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
20:2n-6	0.2 ± 0.0	0.5 ± 0.1**	0.2 ± 0.0	0.3 ± 0.1
20:3n-6	0.3 ± 0.1	1.3 ± 0.3**	0.6 ± 0.1*	2.7 ± 0.3*,**
20:4n-6	33.5 ± 4.8	30.8 ± 4.3	33.9 ± 1.8	26.5 ± 4.8**
22:4n-6	1.0 ± 0.1	0.7 ± 0.2**	0.4 ± 0.1*	0.3 ± 0.1*
22:5n-6	5.6 ± 1.2	1.3 ± 0.4**	0.8 ± 0.3*	0.4 ± 0.2*
22:6n-3	0.9 ± 0.2	0.6 ± 0.1	10.1 ± 1.4*	6.9 ± 1.9*,**
20:3n-6/20:4n-6 (×100)	0.84 ± 0.08	4.31 ± 0.55**	1.88 ± 0.13*	10.81 ± 1.12*,**

Values are means ± S.D.

Results are shown as mg/100 mg total fatty acids present.

*Significantly different than the corresponding young group ($p < 0.05$).

**Significantly different than the age-matched group without cholesterol feeding ($p < 0.05$).

TABLE 5

Fatty Acid Composition of Platelet Phospholipids in Rats With or Without Cholesterol (CHO) Feeding

	11 Weeks of age		64 Weeks of age	
	CHO(−)	CHO(+)	CHO(−)	CHO(+)
Number	7	7	6	7
Fatty acid				
16:0	26.0 ± 2.4	26.1 ± 0.8	23.5 ± 4.2	24.1 ± 1.9
16:1n-7	1.3 ± 0.3	1.6 ± 0.3	1.5 ± 0.6	1.9 ± 0.5
18:0	9.5 ± 3.0	7.9 ± 3.1	7.9 ± 0.6	7.8 ± 0.6
18:1n-9	5.6 ± 0.5	6.6 ± 0.6**	6.7 ± 0.6*	8.0 ± 0.5*,**
18:2n-6	10.7 ± 1.2	13.8 ± 2.5**	9.9 ± 1.0	14.1 ± 2.4**
20:2n-6	0.7 ± 0.2	0.8 ± 0.2	0.4 ± 0.1	0.6 ± 0.1
20:3n-6	0.5 ± 0.2	1.6 ± 0.3**	0.9 ± 0.2*	2.5 ± 0.2*,**
20:4n-6	38.8 ± 2.7	34.2 ± 3.7**	39.3 ± 2.8	33.4 ± 2.3**
20:5n-3	—	0.0 ± 0.0	0.3 ± 0.0	0.5 ± 0.3*
22:4n-6	4.5 ± 1.1	3.8 ± 0.9	3.8 ± 0.6	3.0 ± 0.7
22:5n-6	1.1 ± 0.4	0.3 ± 0.3**	0.2 ± 0.4*	0.2 ± 0.2
22:5n-3	0.1 ± 0.2	0.1 ± 0.1	0.5 ± 0.1*	0.6 ± 0.2*
22:6n-3	0.0 ± 0.1	—	1.1 ± 0.3*	0.9 ± 0.2
20:3n-6/20:4n-6 (×100)	1.35 ± 0.16	4.61 ± 0.46**	2.20 ± 0.17*	7.47 ± 0.47*,**

Values are means ± S.D.

Results are shown as mg/100 mg total fatty acids present.

*Significantly different than the corresponding young group ($p < 0.05$).

**Significantly different than the age-matched group without cholesterol feeding ($p < 0.05$).

TABLE 6

Fatty Acid Composition of Aorta Phospholipids in Rats With or Without Cholesterol (CHO) Feeding

	11 Weeks of age		64 Weeks of age	
	CHO(−)	CHO(+)	CHO(−)	CHO(+)
Number	7	7	6	7
Fatty acid				
16:0	16.2 ± 1.8	17.0 ± 1.4	17.6 ± 0.8	15.3 ± 3.2
16:1n-7	3.8 ± 1.1	2.8 ± 1.1	2.7 ± 0.8	3.1 ± 1.9
18:0	12.0 ± 2.2	13.7 ± 1.6	13.8 ± 0.9	13.3 ± 1.5
18:1n-9	16.4 ± 1.4	14.8 ± 3.7	14.4 ± 0.3	15.8 ± 1.9
18:2n-6	25.0 ± 5.3	22.4 ± 7.8	18.1 ± 4.1	18.6 ± 2.5
20:2n-6	0.4 ± 0.1	0.6 ± 0.1**	0.4 ± 0.1	0.5 ± 0.1
20:3n-6	0.7 ± 0.1	1.6 ± 0.3**	0.8 ± 0.1	1.6 ± 0.2**
20:4n-6	17.7 ± 3.5	18.0 ± 2.0	21.8 ± 2.0	21.3 ± 5.3
20:5n-3	—	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.1
22:4n-6	3.3 ± 0.6	3.8 ± 0.6	3.1 ± 0.5	3.1 ± 0.7
22:5n-6	2.0 ± 0.1	1.5 ± 0.3**	0.7 ± 0.2*	0.8 ± 0.2*
22:5n-3	0.3 ± 0.1	0.3 ± 0.2	0.8 ± 0.2*	1.0 ± 0.1*
22:6n-3	0.2 ± 0.1	0.4 ± 0.4	3.2 ± 0.7*	3.3 ± 1.0*
20:3n-6/20:4n-6 (×100)	3.96 ± 0.28	8.53 ± 0.61**	3.87 ± 0.32	7.94 ± 0.58**

Values are means ± S.D.

Results are shown as mg/100 mg total fatty acids present.

*Significantly different than the corresponding young group ($p < 0.05$).

**Significantly different than the age-matched group without cholesterol feeding ($p < 0.05$).

feeding on fatty acid compositions were only small, and were obtained only in 11-week-old rats (Table 7). 18:1n-9 and 18:3n-3 PUFA were increased and 18:2n-6 PUFA was decreased in 64-week-old rats, in comparison with 11-week-old rats. Marked elevation of the 18:1n-9/18:2n-6 ratio was shown, regardless of cholesterol supplementation.

There were no significant correlations between the plasma cholesterol level and individual fatty acid concentrations in the various tissues in any group of animals or in the whole population. However, in all the tissues in the whole population, the plasma cholesterol was correlated significantly with the 20:3n-6/20:4n-6 ratio (liver, $r = 0.58$, $p < 0.01$; platelets, $r = 0.58$, $p < 0.01$; aorta, $r = 0.43$, $p < 0.05$). Positive but nonsignificant correlations were obtained between plasma cholesterol and the 18:2n-6/20:4n-6 ratios in liver ($r = 0.36$) and platelets ($r = 0.35$).

DISCUSSION

It has been postulated that blood vessel wall/platelet interaction plays a crucial role in the development of atherosclerosis and thrombosis (21,22). Much research on PUFA metabolism in platelets and vascular cells has been performed, because these cells produce biologically active prostaglandins and thromboxanes. Changes in cellular PUFA composition may affect agonist-antagonist reactivity through modifying membrane lipid structure and eicosanoid precursor levels. The PUFA almost certainly have major roles in membranes that are independent of their conversion to eicosanoids. The present study showed that age, which is a risk factor for atherogenesis that cannot be manipulated, and dietary cholesterol, which is a well-known dietary risk factor, substantially affected PUFA compositions in the liver and platelet phospholipids. It has been reported that synthesis of 20:4n-6 from 18:2n-6 is suppressed in the growing rat liver by dietary cholesterol (16). Increased 18:2n-6 and 20:3n-6 levels and 20:3n-6/20:4n-6 ratio in liver phospholipids of 11-week-old and 64-week-old rats, as a result of cholesterol feeding, support this idea. However, in this study, 20:4n-6 was decreased significantly only in 64-week-old rats (Table 4). The positive correlations between plasma cholesterol and the 20:3n-6/20:4n-6 and 18:2n-6/20:4n-6 ratios are consistent with, but do not prove, an inhibitory effect of cholesterol on Δ5- and Δ6-desaturase (16). In platelets, the effects of age and cholesterol were consistent with the results in the liver. PUFA composition of platelets seems to be determined by megakaryocytes in bone marrow (23), and the present study suggests that this process is closely linked with similar PUFA changes in the liver. In contrast, 20:3n-6 content and 20:3n-6/20:4n-6 ratio in aorta phospholipids were unaffected by age, while clearly augmented by dietary cholesterol. Vascular endothelial cells have desaturase and elongation enzyme activities (24,25), that by their own actions might regulate the composition of their fatty acids in diverse ways in response to different modulating factors. The fatty acids in adipose tissue were less affected by cholesterol. However, increase of 18:1n-9 and decrease of 18:2n-6 and subsequent increase of 18:1n-9/18:2n-6 ratio were observed with age. This may be due to increased requirement for PUFA (18:2n-6) with age or may be a reflection of increased 18:1n-9 content as a storage fat pool with age.

TABLE 7

Fatty Acid Composition of Adipose Tissue Lipids in Rats With or Without Cholesterol (CHO) Feeding

	11 Weeks of age		64 Weeks of age	
	CHO(−)	CHO(+)	CHO(−)	CHO(+)
Number	7	7	6	7
Fatty acid				
14:0	1.2 ± 0.1	1.1 ± 0.3	1.2 ± 0.1	1.3 ± 0.2
16:0	18.8 ± 2.2	15.1 ± 2.6**	18.6 ± 2.8	18.3 ± 1.0
16:1n-7	5.2 ± 0.9	6.0 ± 2.4**	7.8 ± 1.9	7.7 ± 1.8
18:0	2.1 ± 0.3	1.6 ± 0.2	1.6 ± 0.6	1.9 ± 0.3
18:1n-9	18.6 ± 0.6	18.2 ± 1.4	32.5 ± 2.9*	30.6 ± 1.7*
18:2n-6	50.9 ± 3.7	58.7 ± 12.7	35.4 ± 1.7*	36.7 ± 3.4*
18:3n-6	—	0.2 ± 0.1	—	—
18:3n-3	—	0.0 ± 0.0	0.4 ± 0.2	0.5 ± 0.2*
20:2n-6	0.4 ± 0.4	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.4
20:3n-6	0.4 ± 0.8	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
20:4n-6	1.3 ± 0.3	1.0 ± 0.3	0.7 ± 0.2*	0.5 ± 0.1*
22:4n-6	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1*	—
22:5n-6	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0*	—
22:5n-3	—	—	0.2 ± 0.1	0.3 ± 0.1
22:6n-3	—	—	0.3 ± 0.2	0.4 ± 0.1
18:1n-9/18:2n-6	0.37 ± 0.02	0.34 ± 0.02	0.92 ± 0.05*	0.84 ± 0.05*

Values are means ± S.D.

Results are shown as mg/100 mg total fatty acids present.

*Significantly different than the corresponding young group ($p < 0.05$).

**Significantly different than the age-matched group without cholesterol feeding ($p < 0.05$).

20:4n-6 is a principal PUFA in the phospholipid fraction and is both a lipid structural component and a precursor for prostaglandin and thromboxane synthesis. Proportions of 20:4n-6 in platelet and aorta phospholipids did not change with increasing age. 20:3n-6 content and 20:3n-6/20:4n-6 ratio in liver and platelet phospholipids were increased with age, suggesting a possible decline of Δ5-desaturase activity, or alternatively, reduced conversion of 20:3n-6 to 1 series prostaglandins. If formation of 20:4n-6 was reduced by age, it must have been compensated by utilizing other 20:4n-6 supplementary pools. 22:5n-6 can be saturated to 22:4n-6 by 4-enoyl-CoA reductase (26), and 22:4n-6 can act as pools for 20:4n-6 formation (8,27), with 22:4n-6 being shortened to 20:4n-6 in the liver peroxisomes (28). An age-dependent increase of peroxisomal C-22 n-6 fatty acids retroconversion, decreased elongation enzyme activity or enhanced incorporation of 20:4n-6 into platelet membrane may be mechanisms for maintaining the 20:4n-6 level constant.

The retention of n-3 PUFA, especially the C-22 fatty acids, was found in all the tissues examined in 64-week-old rats. Whether reduction of n-3 PUFA in 11-week-old rats is a simple reflection of dietary 18:2n-6-induced dilution is uncertain, but there is evidence of an age-dependent increase of 22:6n-3 in brains and other tissues of humans, mice and rats (29–32). N-3 PUFA may turn over at a low rate, a fact which may imply physiological significance. Deficiency of n-3 PUFA has been reported to lead to different kinds of pathological conditions (33,34), including neurological deficits (35), disturbed learning capacity (36), reduced 5′-mononucleotidase in brain (37), visual loss (38) and decreased electrical responses of photoreceptors (39). These phenomena are thought to be related to the normally very high concentrations of 22:6n-3 in the cerebral cortex and in the retina (33,40).

In summary, PUFA levels in platelet phospholipids were affected with age, but 20:4n-6 content was kept constant, though coupled with an increase in 20:3n-6 and a decrease in C-22 n-6 PUFA. The aorta was invulnerable to the effect of age, but was affected by dietary cholesterol, probably due to different responses of enzyme activities for both PUFA synthesis and deacylation-reacylation. The biochemical mechanism of the retention of C-22 n-3 PUFA with age is unknown. Differential utilization of C-22 n-6 and n-3 PUFA requires further investigation in relation to their physiological roles.

REFERENCES

1. Sprecher, H. (1981) *Progr. Lipid Res. 20,* 13–22.
2. Brenner, R.R. (1981) *Progr. Lipid Res. 20,* 41–47.
3. Horrobin, D.F. (1981) *Med. Hypotheses 7,* 1211–1220.
4. Horrobin, D.F. (1982) in *Clinical Uses of Essential Fatty Acids* (Horrobin, D.F., ed.), pp. 3–36, Eden Press, Montreal, Canada.
5. Dilman, V.M. (1981) in *The Law of Deviation of Homeostasis and Diseases of Aging* (Blumenthal, H.T., ed.), pp. 91–128, John Wright PSG, Boston, MA.
6. Blumenthal, H.T. (1983) in *Handbook of Diseases of Aging* (Blumenthal, H.T., ed.), pp. 3–44, Van Nostrand Reinhold Co., New York, NY.
7. Peluffo, R.O., Ayala, S., and Brenner, R.R. (1970) *Am. J. Physiol. 218,* 669–673.
8. Ayala, S., Gaspar, G., Brenner, R.R., Peluffo, R.O., and Kunau, W. (1973) *J. Lipid Res. 14,* 296–305.
9. Moncada, S., and Vane, J.R. (1979) *Pharmacol. Rev. 30,* 293–331.
10. Falardeau, P., Hamberg, M., and Samuelsson, B. (1976) *Biochim. Biophys. Acta 441,* 193–200.

11. Needleman, P., Raz, A., Minkes, M.S., Ferrendelli, J.A., and Sprecher, H. (1979) *Proc. Natl. Acad. Sci. USA 76,* 944-948.
12. Morita, I., Takahashi, R., Saito, Y., and Murota, S-I. (1983) *J. Biol. Chem. 258,* 10197-10199.
13. Bordet, J-C., Guichardant, M., and Lagarde, M. (1986) *Biochem. Biophys. Res. Commun. 135,* 403-410.
14. Horrobin, D.F., and Manku, M.S. (1983) *Lipids 18,* 558-562.
15. Hegsted, D.M., McGandy, R.B., Myers, M.L., and Stare, F.J. (1965) *Am. J. Clin. Nutr. 17,* 281-295.
16. Huang, Y-S., Manku, M.S., and Horrobin, D.F. (1984) *Lipids 19,* 664-672.
17. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
18. Manku, M.S., Horrobin, D.F., Huang, Y-S., and Morse, N. (1983) *Lipids 18,* 906-908.
19. Stewart, J.C.M. (1980) *Anal. Biochem. 104,* 10-14.
20. Sokal, R.R., and Rohlf, F.J. (1981) in *Biometry* (Sokal, R.R., and Rohlf, F.J., eds.), pp. 208-270, W.H. Freeman, New York, NY.
21. Ross, R., and Glomsett, J.A. (1976) *New Engl. J. Med. 295,* 369-377.
22. Gowans, J.L. (1980) in *Blood Cells and Vessel Walls: Functional Interactions* (Ciba Foundation Symposium), pp. 113-26, Excerpta Medica, NY.
23. Schick, P.K., Schick, B.P., Foster, K., and Block, A. (1984) *Biochim. Biophys. Acta 795,* 341-347.
24. Rosenthal, M.D., and Whitehurst, M.C. (1983) *Biochim. Biophys. Acta 750,* 490-496.
25. Rosenthal, M.D. (1987) *Biochim. Biophys. Acta 917,* 279-289.
26. Kunau, W-H., and Bartnik, F. (1974) *Eur. J. Biochem. 48,* 311-318.
27. Sprecher, H. (1967) *Biochim. Biophys. Acta 144,* 296-304.
28. Hagve, T-A., and Christophersen, B.O. (1986) *Biochim. Biophys. Acta 875,* 165-173.
29. Svennerholm, L. (1968) *J. Lipid Res. 9,* 570-579.
30. Sun, G.Y., and Yau, T.M. (1976) *J. Neurochem. 26,* 291-295.
31. Yatsu, F., and Moss, S. (1971) *J. Neurochem. 18,* 1895-1901.
32. Gudbjarnason, S., and Hallgrimsson, T. (1976) *Acta Med. Scand. Suppl. 587,* 17-27.
33. Tinoco, J., Babcock, R., Hincenbergs, I., Medwadowski, B., Miljanich, P., and Williams, M.A. (1979) *Lipids 14,* 166-173.
34. Rudin, D.O. (1982) *Med. Hypotheses 8,* 17-47.
35. Holman, R.T., Johnson, S.B., and Hatch, T.F. (1982) *Am. J. Clin. Nutr. 35,* 617-623.
36. Lamptey, M.S., and Walker, B.L. (1976) *J. Nutr. 106,* 86-93.
37. Bernsohn, J., and Spitz, F.J. (1974) *Biochem. Biophys. Res. Commun. 57,* 293-298.
38. Neuringer, M., Connor, W.E., Van Petten, C., and Barstad, L. (1984) *J. Clin. Invest. 73,* 272-276.
39. Wheeler, T.G., Benolken, R.M., and Anderson, R.E. (1975) *Science 188,* 1312-1314.
40. Anderson, R.E. (1970) *Exp. Eye Res. 10,* 339-344.

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Accumulation of (n-9)-Eicosatrienoic and Docosatrienoic Acids in Human Fibroblast Phospholipids[1]

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An essential fatty acid (EFA) deficiency-like profile of fatty acids has been observed in HF-1 human skin fibroblasts cultured at clonal densities in MCDB 110 and 0.4% fetal bovine serum (FBS). The profile was characterized by an accumulation of 16:1n-7, 18:1n-9, 20:3n-9 and 22:3n-9, a reduction of n-6 fatty acids and a reduction in total polyunsaturated fatty acids (PUFA). The fatty acid composition of sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) was determined and, except for SPH, each displayed an EFA deficiency-like profile. The triene to tetraene ratio (20:3n-9/20:4n-6) ranged from 5.3 in PI to 0.9 in PE. In addition, the highest percentage of 20:3n-9 was present in the PI and the highest percentage of 22:3n-9, in PE. Other human fibroblasts (normal, transformed and at different population doubling number levels [PDL]) were grown under the same conditions and were found to display triene to tetraene ratios (20:3n-9/20:4n-6) in total cellular lipids ranging from 0.7 to 4.5. The accumulation of 20:3n-9 and 22:3n-9 is due primarily to the existence of a basal nutrient medium (MCDB 110) that allows for the rapid clonal growth of human fibroblasts at reduced serum levels (0.4%). This culture procedure can be exploited to further elucidate various aspects of lipid metabolism in human fibroblasts. ***Lipids 23,*** **891–898 (1988).**

A consistent biochemical feature of n-6 fatty acid deficiency in mammals fed a fat-free diet is the reduction of n-6 fatty acids and the accumulation of 20:3n-9 and other 18:1n-9 metabolites in the cellular lipids (1–3). Clinical assessment of essential fatty acid (EFA) status is based most frequently on the triene to tetraene ratio (20:3n-9/20:4n-6) in tissue or plasma lipid fractions (4,5). The 20:3n-9 accumulates in various lipid classes, the extent and pattern of which depend on species, age and sex of the animal, length and severity of the deficiency, and tissue and/or subcellular fraction analyzed within a tissue (6–9).

Linoleic acid or other n-6 polyunsaturated fatty acids (PUFA) have not been proven to be essential nutrients for the multiplication of mammalian cells in culture (10–13). The mass accumulation of 20:3n-9 and related fatty acids coming from de novo synthesis has been described for mammalian cells in culture under rare and/or unusual circumstances. Bailey et al. (14) describe the adaptation of monolayer cultures of MB III cells (mouse lymphoma) to the lipid free medium 7CLF. After seven wk under these culture conditions, the cell monolayer accumulated 20:3n-9 and 22:3n-9, as 29.0% and 42.6% of their total cell fatty acids, respectively. A similar phenomenon occurred with Chang liver cells (human, transformed), but not with mouse L fibroblasts (transformed). Rat embryo fibroblasts do not accumulate 20:3n-9 in cellular phospholipids when cultured in minimum essential medium (MEM) plus 10% tryptose phosphate broth and 10% fetal bovine serum (FBS); however, herpes simplex virus-transformed rat embryo fibroblasts accumulate 20:3n-9 in phospholipid under these culture conditions (15). Hyman et al. (16) have described the accumulation of 20:3n-9 in the phospholipids of mouse 3T3 fibroblasts (ATCC CCL 92 and 92.1) (heteroploid, some transformed characteristics) cultured as confluent monolayers in Dulbecco-modified Eagle medium (DME) plus 10% FBS. Although a variety of other transformed cell types produce lesser amounts of 20:3n-9, human skin fibroblasts (normal diploid) or endothelial cells (normal diploid) do not accumulate this fatty acid under these conditions (17,18). Balb c/3T3 cells and SV-40 transformed Balb c/3T3 cells also accumulate 20:3n-9 during multiplication in a modified MEM supplemented with 10% FBS. The content of 20:3n-9 increases when FBS is replaced with delipidized FBS (19). Rosenthal and Whitehurst (20) describe the accumulation of 20:3n-9 and 22:3n-9 in the total cellular lipids of GM-10 human fetal skin fibroblasts (normal diploid) from log phase cultures of cells grown in MEM plus 5% delipidized bovine serum over an eight-day period. The GM-10 fibroblasts were apparently selected for their ability to multiply rapidly under these conditions, that would normally inhibit the rate of multiplication of other human (21,22) and mouse (19,23) fibroblast cell lines. Laposta et al. (24), after adaptation in Ham's F-10 plus 4.2% delipidized horse serum, generated an EFD-1 cell line with epithelial morphology from HSDM$_1$C mouse fibroblast (transformed). The cells accumulate 20:3n-9 in total cellular lipid under high-density growth conditions in F-10 plus delipidized serum. Primary cultures of mouse brain cells cultured in medium supplemented with 10% FBS (25) and rat normal and transformed glial cells (26), cultured in DME supplemented with 10 and 5% FBS, respectively, accumulate 20:3n-9 in cellular phospholipid.

The experiments in this communication describe the production of an EFA deficient-type fatty acid profile in the phospholipids of a wide variety of human fibroblasts undergoing rapid multiplication in culture. A detailed fatty acid analysis of cellular phospholipid is undertaken to accurately define the accumulation of 20:3n-9 and 22:3n-9 in the structural lipids of the cells.

[1]Fatty acids are abbreviated as number of carbon atoms:number of double bonds, followed by an n-number to designate the position of the first double bond with respect to the methyl carbon. Thus, Mead acid is 20:3n-9 and its elongation product is 22:3n-9.

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Abbreviations: ANS, 8-anilino-1-naphthalene-sulfonic acid; BHT, butylated hydroxytoluene; CS_2, carbon disulfide; EFA, essential fatty acid; FAME, fatty acid methyl ester; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SPH, sphingomyelin; DME, Dulbecco-modified Eagle medium; FBS, fetal bovine serum; GLC, gas liquid chromatography; MECL, modified equivalent chain length; MEM, minimum essential medium; PDL, population doubling number level; TLC, thin layer chromatography.

MATERIALS AND METHODS

Cells. HF-1 and HF-2 are human fibroblasts from newborn foreskins generated in this laboratory by primary culture (27). Flow 2,000 human fibroblasts were purchased from Flow Laboratories (Mississauga, Ontario). WI-38, WI-26VA4 and WI-38VA13 human fibroblasts were purchased from the American Type Tissue Culture Collection (Bethesda, MD) and GM 6171, GM 637A from Institute for Medical Research (Camden, NJ).

Cell aging. The cells were routinely passed at a 1:5 split ratio in T-75 flasks (Falcon) in DME (Flow Laboratories) containing 10% (v/v) FBS (Flow), 100 mg/l penicillin-G (Sigma, 1670 units/mg), 100 mg/l streptomycin sulfate (Sigma) and 10 mg/l gentamycin sulfate (Sigma). The pH was controlled with 30 mM HEPES (Calbiochem) and adjusted to 7.6. Incubation occurred in a water-jacketed incubator (Forma Scientific) saturated with water vapor and adjusted to 2% CO_2. The age of the cells in culture was calculated using the evaluation of the population doubling number level (PDL) (28).

Cell storage. At the appropriate cumulative PDL, cells were stored in liquid nitrogen for later use. One day prior to storage, the cells were refed with fresh culture medium. The cells were suspended after trypsinization in DME containing 20% FBS, 10% dimethylsulfoxide (Sigma) and the antibiotics mentioned above, and aliquoted in 1.0 ml portions into sterile freezer vials (Fisher). The vials were placed at 4 C for 30 min, −70 C for 4 hr and then stored in liquid nitrogen.

Preparation for clonal growth. MCDB 110 was prepared in this laboratory from individually prepared stocks (29,30). The frozen cells were quickly thawed and their contents introduced into T-75 flasks containing freshly prepared MCDB 110, 10% FBS and the antibiotics previously mentioned for 12–24 hr. At that time, the medium was changed to one containing MCDB 110 and 2% FBS, plus antibiotics, for 48 hr. The medium was changed again to one containing 0.1% serum, without antibiotics, for 24 hr; after this point no antibiotics were introduced into the medium. On the day of the beginning of the growth period, the cells were incubated for ca. six hr in MCDB 110 with no serum supplement.

Clonal growth. The cells were trypsinized by a cold trypsinization technique (31) using 0.005% trypsin in Solution A (30 mM HEPES-NaOH [pH 7.6], 10 mM glucose, 130 mM NaCl, 1.0 mM Na_2HPO_4 and 3 mM KCl). The cells were counted by hemocytometer and inoculated into T-75 flasks at 100 cells/cm² for HF-1 PDL 15, 400 cells/cm² for HF-1 PDL 39 and at 200 cells/cm² for the other cells. The incubation period was 11 days.

Cells were grown in the presence of MCDB 110 containing either 4% serum (serum complete medium), in 8–10 T-75 flasks, or 0.4% serum (serum deficient medium), in 15–20 T-75 flasks, 20 ml final volume. For growth at 0.4% serum, the growth surface of the flasks was coated with poly-D-lysine to improve cell attachment (32).

Cell harvesting. At the end of the growth period, the flasks were removed from the incubator and placed on an ice bath for 15 min, prior to the removal of the medium. The cell layer was washed with three 10-ml portions of ice-cold Solution A and subsequently removed from the surface of the culture flask by scraping with a rubber policeman. The harvested cells were centrifuged at 500 × *g* for 5 min at 4 C and resuspended in a small volume of Solution A. Aliquots were taken for protein determination (33) and lipid analysis.

Lipid analysis. All containers were initially rinsed with chloroform/methanol (2:1, v/v). For the cells, the lipids were extracted by the method of Bligh and Dyer (34), and for the FBS, the lipids were extracted by the method of Folch et al. (35). All lipid extractions were performed in the presence of 10^{-9} M butylated hydroxytoluene (BHT) (Sigma).

Chromatography of phospholipids. An aliquot of the lipid extract was applied to a silica gel 60 thin layer chromatography (TLC) plate (Merck) previously activated at 110 C for 1 hr. The TLC plate was developed with chloroform/methanol/acetic acid/water (50:37.5:4:2, v/v/v/v) (36) containing 0.01% BHT (37). The phospholipids were visualized by a UV light source after spraying with 0.1% 8-anilino-1-naphthalene-sulfonic acid (ANS) (Sigma) (38). Phospholipid standards were purchased from Sigma.

The phospholipids were quantitated by the method described by Christie et al. (39). The mol of each identified fatty acid were determined by initially evaluating its mass by a comparison with an internal standard and then by employing the corresponding molecular weight. The unidentified fatty acids were included in the quantitation of the phospholipids. The mol of the unidentified fatty acids were determined using the weighed average molecular weight of the identified fatty acids. The numbers of mol of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) were determined by dividing the number of mol of fatty acids in each phospholipid by 2, and of sphingomyelin (SPH), the number of mol of fatty acids were divided by 1.

Transmethylation. All methylations, either total lipid or phospholipid in the presence of silica gel, were performed with 3.0 ml of 6% sulfuric acid in methanol for 14 hr at 75–80 C (40) in the presence of a known mass of 17:0 (Nu-Chek Prep., Elysian, MN). After cooling to room temperature, 2.0 ml of hexane were added and the contents vortexed for 20 sec. Then, 1.0 ml of water was added and the contents vortexed again. The upper hexane layer was removed and dried under oxygen-free nitrogen.

The methylation products from phospholipid fractions were redissolved in 20 μl of carbon disulfide (CS_2) and an aliquot applied directly to a gas liquid chromatography (GLC) column for analysis. The methylation products from a total lipid extract, either from fibroblasts or FBS, were redissolved in 20 μl hexane and applied to a TLC plate (Merck) and chromatographed in a tank containing 1,2-dichloroethane (41) for isolation of the fatty acid methyl esters (FAME). The FAME band was visualized by ANS and scraped. The FAME were released from the gel using 2 ml methanol, 2 ml hexane and 1 ml deionized water. The hexane layer was removed and processed as described for the phospholipid fractions.

GLC. The FAME were separated on a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector system, N_2 as make-up gas, with a 30 m DB-255 megabore column (J & W Scientific, Rancho Cordova, CA). The column temperature was maintained at 210 C and inlet and detector temperatures each at 250 C. The column gas was helium at 6.7 ml/min with a 1:3 split.

The FAME were identified by comparison of retention times with those of known standards (Nu-Chek Prep.), except for 20:3n-9 and 22:3n-9. The mass of the FAME were calculated by comparison with the area of the internal standard peak using a Spectra Physics integrator.

Identification of n-9 fatty acids. Three methods were used to identify 20:3n-9 and 22:3n-9 fatty acids in human skin fibroblast phospholipids. First, the lipid extracts of the livers of rats fed a fat-free diet were used as a biological standard. Second, argentation-TLC and GLC with appropriate standards were used to confirm that the unknown fatty acids were trienes. Third, indirect GLC-identification techniques for long-chain unsaturated fatty acids were used. Weanling, male Wistar rats were fed semipurified diets (42) that were either fat-free or supplemented with 5% corn oil. After 30 days, the rats were killed, livers removed and liver lipids extracted (35). Total phospholipids were isolated by TLC (43) and then transmethylated as above. Comparison of the phospholipid fatty acids from the rats fed the fat-free and corn oil-supplemented diets revealed two new peaks from the extracts of the rats fed a fat-free diet—one that was 12.0 ± 0.07 mol% and the other, with a longer retention time, that was 0.30 ± 0.01 mol%. Separation of the triene fatty acids by argentation-chromatography and subsequent GLC revealed that these two fatty acids belonged to the triene class of fatty acids and, thus, were identified as 20:3n-9 and 22:3n-9. These fatty acids have been reported to accumulate in liver phospholipids of EFA-deficient rats at similar concentrations (44–46). Lipid extracts from human fibroblasts cultured in 0.4% serum also contained two new peaks with retention times identical to the 20:3n-9 and 22:3n-9 fatty acids found in the phospholipids of rats fed the fat-free semipurified diet. With argentation-chromatography, these two fatty acids were found to be triene fatty acids. In an attempt to confirm that these two fatty acids belonged to the n-9 family of fatty acids, separation factors and modified equivalent chain lengths (MECL) were employed. The column from which retention times were taken was a 0.53 mm open tubular column 30 m long. The stationary phase was 1.0 μ in thickness and composed of 25% cyanopropyl, 25% phenyl and 50% methyl polysiloxane (Megabore DB-225). The MECL values were evaluated according to Jamieson (47): $MECL = 2[\log R_x - \log R_n/\log R_{n+2} - \log R_n] + n$, where R_x, R_n and R_{n+2} are the retention times of the unknown acid and of the n - 9 monoene acids of chain length n and n + 2. The result of this calculation gave observed values. The graph of log retention time vs the carbon number from 16:1n-9 to 22:1n-9 had a linear regression coefficient equal to 0.999. The MECL of 20:3n-9 and 22:3n-9 were calculated according to Jamieson and Reid (48): $MECL_x = MECL_y - \text{Type } II_{6/9}$, where, for the case of 20:3n-9, x refers to 20:3n-9 and y to 20:4n-6, and for the case of 22:3n-9, x refers to 22:3n-9 and y refers to 22:4n-6, and Type $II_{6/9}$ refers to the difference in MECL values of the pairs of esters used to calculate the Type II separation factors. The result of this calculation gave expected values. The observed and expected values for 20:3n-9 were 20.38 ± 0.01 and 20.37 ± 0.01, and for 22:3n-9, the corresponding values were 22.44 ± 0.01 and 22.42 ± 0.01.

The retention times of 20:3n-9 on our standard (210 C isothermal) GLC analysis results in a significant overlap of the 20:3n-9 and 20:2n-6 peaks. However, human fibroblasts cultured under these conditions have undetectable levels of 20:2n-6 in total phospholipid fatty acids and in each phospholipid fraction except PI (4.0% serum). This was determined by analysis of diene fatty acids after argentation-chromatography and by analysis of fatty acids at 160 C isothermal, which completely separates 20:3n-9 and 20:2n-6, with 20:3n-9 eluting first.

Argentation-chromatography. Eight g of silver nitrate were dissolved in 80 ml of water, to which 40 g of silica gel (CAMAG DS-5) were added, and the contents were poured on 20 × 20 cm glass plates (0.35-mm thick) and allowed to dry. FAME from the various lipid extracts were spiked with 18:3n-3 and chromatographed. The plate was developed in chloroform/methanol (99:1, v/v) and the triene bands scraped, the FAME eluted and analyzed by GLC, as previously described.

Statistical analysis. Data within a phospholipid were analyzed for variance (ANOVA) followed by a Tukey's studentized range test; this was done so that the residuals could be used to test whether the data came from a normal distribution using the Shapiro-Wilk statistic, W. Data were log transformed before analysis, if necessary, to achieve a normal distribution (49).

RESULTS

Human HF-1 fibroblasts cultured at a density of 8×10^3 cells/T-75 flask in MCDB 110 plus 0.4% FBS exhibited significantly less total growth over the 13-day assay period than the cells cultured in MCDB 110 plus 4.0% FBS, 0.99 ± 0.04 vs 1.65 ± 0.11 × 10^6 cells/T-75 flask ($P < 0.05$, $n = 4$), respectively. The effect of culturing HF-1 fibroblasts in MCDB 110 plus 0.4% FBS on the phospholipid profile is shown in Table 1. Compared to the profile of phospholipids found in cells cultured in MCDB 110 plus 4.0% fetal calf serum, cells cultured with 0.4%

TABLE 1

Effect of Serum Concentration on the Distribution of Phospholipids in HF-1 Human Skin Fibroblasts in a Clonal Growth Assay

	Phospholipid distribution % Fetal bovine serum[a]	
Phospholipid	4.0	0.4
	mol%[b,c]	
Sphingomyelin	9.9 ± 0.4	9.0 ± 0.5
Phosphatidylcholine	48.4 ± 1.1	46.1 ± 1.3
Phosphatidylserine	10.1 ± 0.5	9.8 ± 0.6
Phosphatidylinositol	7.9 ± 0.3	8.4 ± 0.4
Phosphatidylethanolamine	23.7 ± 0.7[d]	26.7 ± 0.8

[a]Fetal bovine serum lot #1.

[b]Values are mean ± SEM, n = 6 for 4.0%, n = 5 for 0.4% FBS. The data represent results from 4 independent experiments utilizing fresh media and new cells brought up from frozen stocks. In one experiment, 3 sets of assay flasks were processed for 4.0% serum, 2 for 0.4% serum.

[c]Percent distribution of phospholipids is based on the five phospholipid groups that were analyzed and not on the total phospholipids in the cell.

[d]Values are significantly different by the Tukey's test ($P < 0.05$).

TABLE 2

Effect of Serum Concentration on the Fatty Acid Composition of Sphingomyelin, Phosphatidylinositol, Phosphatidylethanolamine, Phosphatidylcholine and Phosphatidylserine in HF-1 Human Skin Fibroblasts in a Clonal Growth Assay

	% Fetal bovine serum[a]									
	Sphingomyelin		Phosphatidylinositol		Phosphatidylethanolamine		Phosphatidylcholine		Phosphatidylserine	
Fatty acid	4.0	0.4	4.0	0.4	4.0	0.4	4.0	0.4	4.0	0.4
	(mol%)[b]									
14:0	1.4 ± 0.4[c]	5.0 ± 0.5	tr	tr	tr	1.3 ± 0.3	1.5 ± 0.2[c]	4.7 ± 0.2	tr	0.7 ± 0.1
14:1	tr	tr	tr	tr	1.8 ± 0.6	tr	tr	tr	tr	tr
16:0	51.6 ± 1.9	49.9 ± 2.1	9.3 ± 1.8	5.4 ± 1.9	8.1 ± 1.1	9.1 ± 1.2	30.7 ± 0.4[c]	28.5 ± 0.4	12.2 ± 1.2	11.2 ± 1.3
16:1	0.9 ± 0.4	1.4 ± 0.4	2.2 ± 0.2[c]	4.6 ± 0.2	3.2 ± 0.9[c]	7.0 ± 1.0	6.9 ± 0.7[c,g]	18.0 ± 0.7	2.5 ± 0.4[c]	6.3 ± 0.4
18:0	4.5 ± 1.1	2.7 ± 1.2	29.1 ± 1.1	27.0 ± 1.2	15.8 ± 0.5[c]	9.6 ± 0.5	6.5 ± 0.2[c,g]	3.0 ± 0.2	31.8 ± 1.0[c]	27.9 ± 1.1
18:1	1.3 ± 0.6	1.6 ± 0.6	20.3 ± 1.2[c]	28.0 ± 1.3	14.7 ± 1.1[c]	28.7 ± 1.2	30.7 ± 0.4	31.5 ± 0.4	23.7 ± 1.0[c]	30.3 ± 1.1
18:2[d]	1.5 ± 0.5	0.6 ± 0.4	4.3 ± 0.5	3.1 ± 0.6	2.5 ± 0.2	2.5 ± 0.2	3.7 ± 0.2	3.6 ± 0.2	4.0 ± 0.3[c]	2.4 ± 0.4
18:3	ND	ND	ND	ND	tr	tr	tr	ND	tr	tr
20:0	0.9 ± 0.2	0.8 ± 0.2	tr	tr	tr	tr	tr	ND	tr	tr
20:1	tr	tr	tr	tr	tr	0.6 ± 0.04	tr	tr	tr	0.9 ± 0.1
20:3n-9	ND	ND	ND	17.7 ± 1.6	ND	4.0 ± 0.4	ND	2.3 ± 0.2	ND	1.6 ± 0.2
20:3n-6	ND	ND	1.7 ± 0.2[c]	0.6 ± 0.2	0.9 ± 0.1	tr	1.2 ± 0.1	tr	1.9 ± 0.2[c]	0.6 ± 0.2
20:4n-6	tr	tr	23.4 ± 1.4[c]	4.1 ± 1.4	12.5 ± 0.6[c]	5.1 ± 0.6	6.0 ± 0.3[c]	1.0 ± 0.4	2.7 ± 0.1[c]	0.6 ± 0.2
22:0	8.9 ± 0.5[c]	5.2 ± 0.5	ND	tr	tr	tr	ND	ND	1.5 ± 0.3	2.3 ± 0.3
22:1	0.7 ± 0.2	tr	tr	tr	tr	tr	tr	tr	0.6 ± 0.1	0.8 ± 0.2
22:3n-9	ND	ND	ND	1.2 ± 0.1	ND	3.7 ± 0.3	ND	tr	ND	1.6 ± 0.2
22:4n-6	ND	ND	0.8 ± 0.1	tr	4.4 ± 0.4[c]	1.4 ± 0.4	1.0 ± 0.1	tr	2.3 ± 0.1[c]	0.9 ± 0.1
22:5n-3	ND	ND	1.5 ± 0.1	ND	6.4 ± 0.3[c]	1.8 ± 0.3	1.6 ± 0.1	tr	3.2 ± 0.2[c]	1.2 ± 0.2
22:6n-3	ND	ND	tr	tr	6.2 ± 0.5[c]	2.4 ± 0.5	1.2 ± 0.1	tr	2.3 ± 0.2[c]	0.9 ± 0.2
24:0	6.0 ± 0.3	5.4 ± 0.3	ND	tr	tr	tr	tr	tr	1.7 ± 0.2[c]	0.9 ± 0.2
24:1	17.7 ± 0.7	20.0 ± 0.8	ND	tr	tr	0.7 ± 0.2	tr	tr	1.6 ± 0.3	1.8 ± 0.3
Unidentified	3.5 ± 0.7	7.1 ± 2.5	5.8 ± 2.0	6.7 ± 0.6	23.1 ± 0.5	20.1 ± 2.0	7.3 ± 0.2	5.4 ± 0.4	6.9 ± 1.5	6.4 ± 0.4
Fatty acid class										
Saturated	73.1 ± 1.6	68.9 ± 1.8	38.9 ± 1.4[c]	33.0 ± 1.5	24.3 ± 1.1[c,g]	20.9 ± 1.4	39.0 ± 0.5[c]	36.4 ± 0.5	47.8 ± 1.1[c]	43.4 ± 1.2
Monounsaturated	21.4 ± 0.5[c]	23.1 ± 0.5	23.1 ± 1.1[c]	33.2 ± 1.2	20.2 ± 1.7[c]	37.7 ± 1.9	38.1 ± 0.6[c]	50.2 ± 0.6	28.8 ± 1.3[c]	40.0 ± 1.4
Polyunsaturated	2.0 ± 0.3[c]	0.8 ± 0.4	33.1 ± 1.4[c]	27.0 ± 1.4	32.9 ± 1.0[c]	21.4 ± 1.1	15.4 ± 0.6[c]	8.0 ± 0.7	16.8 ± 0.4[c]	10.0 ± 0.4
Double bond index[e]	0.3 ± 0.02	0.3 ± 0.02	1.5 ± 0.1[c]	1.2 ± 0.1	1.6 ± 0.1[c]	1.2 ± 0.1	0.9 ± 0.02	0.7 ± 0.02	0.9 ± 0.02[c]	0.7 ± 0.02
Unsaturated/saturated[f]	0.4 ± 0.02	0.4 ± 0.02	3.8 ± 0.2	3.5 ± 0.3	6.8 ± 0.4	5.6 ± 0.5	2.4 ± 0.1[c]	2.0 ± 0.1	2.0 ± 0.1[c]	1.7 ± 0.1

[a]Fetal bovine serum lot #1. The fatty acid composition of this serum lot is shown in Table 3.

[b]Values are presented as mean ± SEM, n = 6 and 5 for phospholipids at 4.0 and 0.4% serum, respectively. The data represent results from 4 independent experiments utilizing fresh media and new cells brought up from frozen stocks. In one experiment, 3 sets of assay flasks were processed for 4.9% serum, 2 for 0.4% serum.

[c]Values are significantly different by Tukey's studentized range test. Only values within a particular phospholipid are statistically compared.

[d]Represents multiple positional and/or *cis/trans* isomers.

[e]Average number of double bonds per fatty acid in the sample.

[f]Double bond index/mol% of total saturated fatty acids × 100 (8).

[g]This data was log transformed to achieve a normal distribution prior to the application of analysis of variance followed by a Tukey's studentized range test.

Tr, trace (<0.5%); ND, not detectable (<0.1%).

fetal calf serum have significantly more PE. Cells cultured under both conditions have similar total phospholipid (sum of those detected), 323 ± 17 and 300 ± 18 nmol/mg protein ($P > 0.05$) for cells cultured with 0.4 and 4.0% FBS, resepectively.

The fatty acid composition of the phospholipids of HF-1 fibroblasts cultured under the experimental conditions is shown in Table 2. The fatty acid composition of the FBS used to culture these cells is shown in Table 3 (serum lot 1). In SPH, cells cultured with 0.4% FBS have significantly more 14:0 and fewer 20:0, compared with 4.0% controls. The cells also have a significantly higher number of total monounsaturated fatty acids. In PI, cells have significantly fewer 20:3n-6 and 20:4n-6, and 22:4n-6 and 22:5n-3 concentrations drop to trace (<0.5%) and not-detectable (<0.1%) levels, respectively. The cells have significantly more 16:1 and 18:1 and show an accumulation of 20:3n-9 and 22:3n-9. In this phospholipid, cells have significantly fewer total saturated fatty acids, a lower double bond index and more total monounsaturated fatty acids, when cultured with 0.4% vs 4.0% FBS. In PE, cells have significantly fewer 18:0, 20:4n-6, 22:4n-6, 22:5n-3 and 22:6n-3. The cells have significantly more 16:1, 18:1 and fewer 18:0 and show an accumulation of 20:3n-9 and 22:3n-9. The cells have significantly fewer total saturated fatty acids, total PUFA and a lower double bond index and more total monounsaturated fatty acids in PE. In PC, the cells cultured with 0.4% FBS have significantly fewer 16:0, 18:0 and 20:4n-6 and 20:3n-6 and 20:3n-6 falls to trace levels. The cells have significantly more 14:0 and 16:1 and have an accumulation of 20:3n-9 and 22:3n-9. They have fewer total saturated fatty acids, fewer total PUFA, a smaller unsaturated to saturated ratio and greater total monounsaturated fatty acids in this phospholipod. In PS, the cells have significantly fewer 18:0, 18:2 (total isomers), 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-3, 22:6n-3 and 24:0 and significantly more 16:1, 18:1 and 22:0, when cultured in 0.4% FBS. The cells accumulate 20:3n-9 and 22:3n-9 in PS under these conditions. The cells have more total monounsaturated fatty acids, but fewer total saturated PUFA, a lower double bond index and lower unsaturated-to-saturated ratio in this phospholipid under these conditions.

A comparison of the accumulation of 20:3n-9 and 22:3n-9 among the phospholipid fractions of HF-1 cells cultured in MCDB 110 plus 0.4% FBS is shown in Table 4. As a mol% of total fatty acids, 20:3n-9 accumulates in phospholipids as follows: PI > PE > PC = PS; and 22:3n-9 accumulates in phospholipids: PE > PS = PI > PC. However, as a mol% of the total 20:3n-9 found in phospholipids of the cells, 20:3n-9 accumulates as follows: PI > PC = PE > PS; and 22:3n-9 as PE > PC = PS with PS = PI, but PC > PI. In terms of the ratio 20:3n-9/20:4n-6, the cells have the following distribution in phospholipids: PI > PE, but PI = PS = PC and PS = PC = PE. These values are compared by the Tukey's studentized range test and are significantly different at the $P < 0.05$ level, $n = 5$.

The effect of fibroblast cell type on the accumulation of 20:3n-9 in cells cultured in MCDB 110 plus 0.4% FBS is shown in Table 5. All fibroblasts tested showed an accumulation of 20:3n-9, regardless of whether they were of lung or skin origin, normal or transformed, low or high PDL. The mol% of 20:3n-9 in total fatty acids from these cells ranged from 0.8 to 3.7, and 20:3n-9/20:4n-6 ratios ranged from 0.6 to 4.5. None of the cells had any detectable 20:3n-9, when cultured with 4.0% FBS. The lot of FBS used appeared to have a minimal effect on the accumulation of 20:3n-9 in fibroblasts (Tables 3 and 5).

TABLE 3

Fatty Acid Composition of Fetal Bovine Serum

Fatty acid	Serum lot number[a]		
	1	2	3
		(mol%)[b,c]	
14:0	1.8 ± 0.1	1.4 ± 0.1	1.6 ± 0.1
16:0	26.7 ± 0.9	24.6 ± 0.5	26.3 ± 0.2
16:1	5.8 ± 0.1	5.8 ± 0.1	4.9 ± 0.1
18:0	12.0 ± 0.3	11.8 ± 0.1	12.7 ± 0.2
18:1	24.0 ± 0.3	25.7 ± 0.5	21.6 ± 0.3
18:2	4.7 ± 0.1	4.6 ± 0.4	5.2 ± 0.1
18:3	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
20:0	tr	0.6 ± 0.1	0.5 ± 0.1
20:3n-6	1.6 ± 0.1	1.8 ± 0.1	1.6 ± 0.1
20:4n-6	6.1 ± 0.4	5.6 ± 0.1	6.8 ± 0.1
22:0	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
22:4n-6	tr	tr	0.7 ± 0.1
22:5n-3	1.7 ± 0.2	2.1 ± 0.1	1.2 ± 0.1
22:6n-3	2.2 ± 0.2	2.4 ± 0.1	1.8 ± 0.1
24:0	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
24:1	1.6 ± 0.1	1.7 ± 0.1	1.4 ± 0.1
Unidentified	7.9 ± 0.4	8.2 ± 0.3	8.7 ± 1.6

[a]Mean ± SEM of analysis of serum lots; lot 1, n = 4, lots 2 and 3, n = 3.

[b]Mean values below 0.5 mol% are listed as trace (tr) levels.

[c]All serum lots have trace levels (<0.5%) of 20:3n-9 and nondetectable levels of 22:3n-9 (<0.1%).

DISCUSSION

Research in our laboratory has utilized three observations to cultivate rapidly dividing human fibroblasts that have a phospholipid fatty acid composition that closely resembles the fatty acid profile from the tissue of EFA-deficient animals (4,50) and humans (5). First, fibroblasts in culture preferentially utilize exogenously supplied lipids to meet their metabolic needs, however, they are capable of increasing their rates of fatty acid and cholesterol biosynthesis when the culture medium has a low lipid content (11). Second, in rapidly dividing fibroblasts, there is a high demand for lipid material because the cells must assemble a large quantity of membrane lipids as part of the general mitotic process, in addition to the continuous turnover of existing membrane structures (12). Third, the qualitative and quantitative optimization of the concentration of nutrients in a cell-culture medium can reduce the amount of FBS necessary for maximal rates of cell multiplication for a variety of cell types (51). The utilization of MCDB 110 as the culture medium, which has been optimized specifically for the clonal growth of human diploid fibroblasts with low levels of FBS supplementation (29,30), appears to have resulted in cells with rates of multiplication, and thus the need for membrane lipid material, that significantly exceed the rate at which they can take up and utilize lipid from

TABLE 4

Comparison of the Distribution of 20:3n-9 and 22:3n-9 Among Phospholipids in HF-1 Human Fibroblasts in a Clonal Growth Assay at 0.4% Serum

Fatty acid parameter	Phospholipid[a]			
	PI	PS	PC	PE
20:3n-9[e] (mol% of total fatty acids within a phospholipid)	17.8 ± 1.6[b]	1.6 ± 0.2[c]	2.3 ± 0.2[c]	3.7 ± 0.3[d]
20:3n-9[f] (% of total 20:3n-9 among the phospholipids)	39.7 ± 0.8[b]	4.2 ± 0.2[c]	27.8 ± 0.3[d]	28.3 ± 1.2[d]
22:3n-9 (mol% of total fatty acids within a phospholipid)	1.2 ± 0.1[b]	1.6 ± 0.2[b]	0.4 ± 0.02[c]	3.7 ± 0.3[d]
22:3n-9[f] (% of total 22:3n-9 among the phospholipids)	7.0 ± 0.3[b]	10.8 ± 0.8[b,c]	13.6 ± 1.0[c]	68.5 ± 1.5[d]
20:3n-9/20:4n-6	5.3 ± 1.1[b]	2.9 ± 0.6[b,c]	3.0 ± 0.7[b,c]	0.9 ± 0.2[c]

[a]Mean ± SEM, n = 5. Values having a different superscripted letter (b, c, d) are significantly different by Tukey's studentized range test ($P < 0.05$). The data represent results from 4 independent experiments utilizing fresh media and new cells brought up from frozen stocks. In one experiment, 2 sets of assay flasks were processed.

[e]These data were log transformed to achieve a normal distribution prior to the application of analysis of variance followed by Tukey's studentized range test.

[f]Total mol of the fatty acid in a phospholipid/total mol of the fatty acid in all the phospholipids × 100.

TABLE 5

Accumulation of (n-9) Eicosatrienoic Acid in the Total Lipid of Normal and Transformed Human Fibroblasts in a Clonal Growth Assay

Cell line	Tissue of origin	Type[a]	PDL[b]	Serum lot[c]	% Fetal bovine serum				
					20:3n-9		20:4n-6		20:3n-9/20:4n-6
					4.0	0.4	4.0	0.4	0.4
					(mol%)[d]				
HF-1	Skin	N	15	1	ND	3.5 ± 0.2	6.4 ± 0.6	1.6 ± 0.1	2.2 ± 0.2
				2	ND	3.4 ± 0.3	6.0 ± 0.4	1.7 ± 0.3	2.2 ± 0.5
				3	ND	3.5 ± 0.2	7.5 ± 0.3	2.2 ± 0.2	1.6 ± 0.3
			39	1	ND	2.1 ± 0.5	8.5 ± 0.4	2.9 ± 0.6	0.9 ± 0.4
Flow 2000	Lung	N	20	1	ND	1.7 ± 0.2	6.3 ± 1.1	2.9 ± 0.3	0.6 ± 0.1
WI 38	Lung	N	25	1	ND	2.2 ± 0.3	7.1 ± 1.1	2.7 ± 0.4	0.8 ± 0.1
HF-2	Skin	N	13	1	ND	3.5 ± 0.6	6.9 ± 0.6	1.6 ± 0.2	2.3 ± 0.4
GM 6171	Lung	N	11	2	ND	2.3 ± 0.1	4.6 ± 0.1	2.8 ± 0.1	0.8 ± 0.1
WI 26VA4	Lung	T	>200	2	ND	2.7 ± 0.1	3.2 ± 0.1	0.6 ± 0.1	4.5 ± 0.7
WI 38VA13	Lung	T	>200	2	ND	0.8 ± 0.1	3.7 ± 0.8	1.1 ± 0.1	0.7 ± 0.1
GM 0637 B	Skin	T	>200	2	ND	3.7 ± 0.3	4.6 ± 0.6	1.4 ± 0.5	3.9 ± 0.5

[a]N refers to normal diploid fibroblasts, T refers to SV-40 transformed fibroblasts.

[b]Population doubling number at the start of the clonal growth assay. This is an indicator of the number of previous replications in culture. The population doubling number of the transformed cells is unknown.

[c]The fatty acid composition of the different lots of sera are displayed in Table 3.

[d]Values are presented as mean ± SEM, n = 3 except HF-1, PDL 15, n = 4, WI-38 and GM 6171, n = 2. The data represent results from independent experiments utilizing fresh media and new cells brought up from frozen stocks.

ND, not detectable.

medium supplemented with 0.4% FBS. Fibroblasts cultivated under these conditions synthesize PUFA not found in FBS and insert them into phospholipid. Apparently, this response of the cells is necessary to maintain their rate of multiplication in this chemical milieu. The phenomenon occurs in all the human fibroblast cell types tested and appears highly reproducible.

As studied in HF-1 human foreskin fibroblasts, the general response for fibroblasts cultured in MCDB 110 plus 0.4% serum is to replace n-6 PUFA in phospholipids with 20:3n-9 and 22:3n-9. The HF-1 cells exhibit a distinct pattern of incorporation of n-9 PUFA into phospholipid, i.e., 20:3n-9 is incorporated mainly into PI, and 22:3n-9 mainly into PE. Other types of cells in culture exhibit selectivity for the incorporation of endogenous 20:3n-9 into particular phospholipid classes, however, the selectivity apparently depends on cell type and culture conditions (15,16,19,20,24). In HF-1 cells, the 18:2 isomers are conserved largely in each phospholipid class, presumably because of the accumulation of n-9, n-7 and other isomers (19,24,52). This general pattern of fatty acid distribution is characteristic of EFA deficiency in whole animals (2,45). The 20:3n-9/20:4n-6 ratios observed in human fibroblasts cultured under these conditions ranged from 0.6 to 4.5, and these ratios are greater than the value of 0.2 that has been proposed as the biochemical index of EFA deficiency in human tissue (53). The change in phospholipid composition seen in the HF-1 cells, namely to significantly increase PE, the phospholipid with the highest PUFA, has not been described in tissue from EFA-deficient animals and may be unique to fibroblasts under these culture conditions.

It cannot be determined from these experiments whether human fibroblasts in culture have a requirement for n-6 fatty acids for maximal rates of cell multiplication. In animals with EFA deficiency, the double bond index (degree of unsaturation) of liver fatty acids does not progressively decrease from an index of 1.2–1.3 (4) with increasing time on a fat-free diet, but levels off to approximately 0.8 and is maintained (2). Interestingly, the HF-1 fibroblasts described in these experiments have a double bond index in their total phospholipid fatty acids of 0.84 $\pm$ 0.02, compared with 1.08 $\pm$ 0.02 ($P < 0.05$) for controls. The normal human diploid fibroblasts listed in Table 5 had double bond indices ranging from 0.76 (HF-1 PDL 39) to 0.87 (GM 6171) in their total cellular fatty acids when cultured in MCDB 110 plus 0.4% FBS. The average double bond index of the normal cell types under these conditions was 0.82 $\pm$ 0.02. Of the five phospholipids analyzed, only the unsaturated/saturated ratios (8) of PC and PS were significantly greater for cells grown at 4.0% serum compared with cells grown at 0.4% serum. In terms of total phospholipid fatty acids, the cells cultured in 0.4% serum have a small but statistically significant decrease (2.9 $\pm$ 0.1 vs 3.3 $\pm$ 0.1, $P < 0.05$) in the unsaturated/saturated ratio. It appears that normal human fibroblasts in culture may accumulate n-9 and n-7 monoenes, n-9 and n-7 dienes and trienes, and alter their phospholipid composition to maintain a minimal level of the degree of unsaturation of membrane fatty acids. It is not known whether human fibroblasts can attain maximal rates of cell multiplication while maintaining a double bond index of ca. 0.8 or a slightly reduced unsaturated/saturated ratio. The reduced multiplication rate of human fibroblast cells cultured in MCDB 104 or 110 at $\leq$0.4% serum cannot be overcome by supplementation with linoleate (51,54).

Though the procedure outlined in this communication may not establish a n-6 fatty acid requirement for the proliferation of fibroblasts in culture, it allows for the elucidation of various aspects of their lipid metabolism.

REFERENCES

1. Odes-Jorgensen, E. (1961) *Physiol. Rev. 41,* 1-51.
2. Holman, R.T. (1968) *Progress in the Chemistry of Fats and Other Lipids* (Holman, R.T., ed.) Vol. 9, pp. 275-348, Pergamon Press Ltd., London, England.
3. Yamanaka, W.K., Clemans, G.W., and Hutchinson, M.L. (1981) *Prog. Lipid Res. 19,* 187-215.
4. Holman, R.T. (1978) *CRC Handbook Series in Nutrition and Food, Section E, Nutritional Disorders* (Recheigl, M. Jr., ed.) Vol. II, pp. 491-515, CRC Press, West Palm Beach, FL.
5. Holman, R.T. (1978) *CRC Handbook Series in Nutrition and Food, Section E, Nutritional Disorders* (Recheigl, M. Jr., ed.) Vol. III, pp. 335-368, CRC Press, West Palm Beach, FL.
6. Lefkowith, J.B., Flippo, V., Sprecher, H., and Needleman, P. (1985) *J. Biol. Chem. 260,* 15736-15744.
7. Trugnan, G., Thomas-Benhamou, G., Cardot, P., Rayssiguier, Y., and Bereziat, G. (1985) *Lipids 20,* 862-868.
8. Brenner, R.R., Garda, H., De Goméz Dumm, I.N.T., and Pezzano, H. (1981) *Prog. Lipid Res. 20,* 315-321.
9. Ramesha, C.S., and Picket, W.C. (1987) *J. Lipid Res. 28,* 326-331.
10. Bailey, J.M., and Dunbar, L.M. (1973) *Exp. Mol. Pathol. 18,* 142-161.
11. Spector, A.A., Mathur, S.N., Kaduce, T.L., and Hyman, B.T. (1981) *Prog. Lipid Res. 19,* 155-186.
12. Rosenthal, M.D. (1987) *Prog. Lipid Res. 26,* 87-124.
13. Bettger, W.J., and Ham, R.G. (1982) *Adv. Nutr. Res. 4,* 249-286.
14. Bailey, J.M., Howard, B.V., Dunbar, L.M., and Tillman, S.F. (1972) *Lipids 7,* 125-134.
15. Daniel, L.W., Kucera, L.S., and Waite, M. (1980) *J. Biol. Chem. 255,* 5697-5702.
16. Hyman, B.T., Stoll, L.L., and Spector, A.A. (1981) *J. Biol. Chem. 256,* 8863-8866.
17. Spector, A.A., Kiser, R.E., Denning, G.M., Koh, S.M., and DeBault, L.E. (1979) *J. Lipid Res. 20,* 536-547.
18. Spector, A.A., Hoak, J.C., Fry, G.L., Denning, G.M., Stoll, L.L., and Smith, J.B. (1980) *J. Clin. Invest. 65,* 1003-1012.
19. Tombaccini, D., Fallani, A., Mugnai, G., and Ruggieri, S. (1981) *J. Lipid Res. 22,* 590-597.
20. Rosenthal, M.D., and Whitehurst, M.C. (1982) *J. Cell. Physiol. 113,* 298-306.
21. McKeehan, W.L., and Ham, R.G. (1978) *In Vitro 14,* 353 (abst.).
22. Spector, A.A., and Yorek, M.A. (1986) *J. Lipid Res. 26,* 1015-1035.
23. Hatten, M.E., Horwitz, A.F., and Burger, M.M. (1977) *Exp. Cell Res. 107,* 31-34.
24. Laposta, M., Prescott, S.M., Bross, T.E., and Majerus, P.W. (1982) *Proc. Natl. Acad. Sci. USA 79,* 7654-7658.
25. Bourre, J.M., Faivre, A., Dumont, O., Nouvelot, A., Loudes, C., Peymirat, J., and Tixier-Vidal, A. (1983) *J. Neurochem. 41,* 1234-1242.
26. Robert, J., Montaudon, D., and Hughes, P. (1983) *Biochim. Biophys. Acta 752,* 383-395.
27. Kirkland, J.B., Bray, T.M., and Bettger, W.J. (1987) *Can. J. Physiol. Pharmacol. 65,* 1788-1792.
28. Cristofalo, V.J., and Charpentier, R. (1980) *J. Tissue Culture Methods 6,* 117-121.
29. Bettger, W.J., Boyce, S.T., Walthall, B.J. and Ham, R.G. (1981) *Proc. Natl. Acad. Sci. USA 78,* 5588-5592.
30. McKeehan, W.L., McKeehan, K.A., Hammond, S.L., and Ham, R.G. (1977) *In Vitro 13,* 399-416.
31. McKeehan, W.L. (1977) *Cell Biol. Int. Rep. 1,* 335-343.
32. McKeehan, W.L., and Ham, R.G. (1976) *J. Cell Biol. 71,* 727-734.
33. Peterson, G.L. (1977) *Anal. Biochem. 83,* 346-356.
34. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.

35. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
36. Holub, B.J., and Skeaff, C.M. (1987) *Methods in Enzymology* (Conn, P.M., and Means, A.R., eds.) Vol. 141, pp. 234-244, Academic Press, Inc., Orlando, FL.
37. Neudoerffer, T.S., and Lea, C.H. (1966) *J. Chromatogr. 21,* 138-140.
38. Lynch, R.D. (1980) *Lipids 15,* 412-420.
39. Christie, W.W., Noble, R.C., and Moore, J.H. (1970) *Analyst 95,* 940-944.
40. Mahadevappa, V.G., and Holub, B.J. (1982) *Biochim. Biophys. Acta 713,* 73-79.
41. Kramer, J.K.C., and Halan, H.W. (1976) *J. Lipid Res. 17,* 674-676.
42. Bettger, W.J. (1985) *Nutr. Res. 5,* 1153-1159.
43. Mahadevappa, V.G., and Holub, B.J. (1984) *J. Biol. Chem. 259,* 9369-9373.
44. Mohrhauer, H., and Holman, R.T. (1965) *J. Am. Oil Chem. Soc. 42,* 639-643.
45. Gidez, L.I. (1964) *Biochem. Biophys. Res. Commun. 14,* 413-418.
46. Nervi, A.M., and Brenner, R.R. (1965) *Biochim. Biophys. Acta 106,* 205-207.
47. Jamieson, G.R. (1975) *J. Chromatogr. Sci. 13,* 491-497.
48. Jamieson, G.R., and Reid, E.H. (1969) *J. Chromatogr. 39,* 71-74.
49. SAS Institute Inc. (1985) *SAS User's Guide, Version 5 Edition,* SAS Institute Inc., Box 8000, Cary, NC 27511-8000, U.S.A.
50. Collins, F.D. (1962) *Biochem. Biophys. Res. Commun. 9,* 289-292.
51. Ham, R.G., and McKeehan, W.L. (1978) *In Vitro 14,* 11-22.
52. Sand, D., Sen, N., and Schlenk, H. (1965) *J. Am. Oil Chem. Soc. 42,* 511-516.
53. Holman, R.T. (1976) *Adv. Exp. Med. Biol. 83,* 515-534.
54. Skeaff, S.A., Karmiol, S., and Bettger, W.J. (1985) *Can. Fed. Proc. 28,* 181 (abst.).

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Effects of Aurothioglucose on Iron-induced Rat Liver Microsomal Lipid Peroxidation

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Aurothioglucose (ATG), an inhibitor of selenium-dependent glutathione peroxidase activity, at a concentration of 100 μM, strongly increases lipid peroxidation of rat liver microsomes exposed to either ferrous ion (10 μM) or the combination of ferric ion (10 μM) and ascorbic acid (500 μM), in the presence of reduced glutathione (GSH, 800 μM). This effect was not achieved using heat-inactivated microsomes and was dependent on the presence of GSH. ATG did not affect the lag period associated with ascorbic acid/ferric ion-induced microsomal lipid peroxidation (previously attributed to an undefined GSH-dependent microsomal agent), but did increase the rate of peroxidation subsequent to the lag period. The potent GSH-dependent inhibition of microsomal lipid peroxidation by cytosol (10% of total volume) was completely reversed by ATG (100 μM). ATG similarly reversed an inhibition of phosphatidylcholine hydroperoxide-dependent liposomal peroxidation that has been attributed to phospholipid hydroperoxide glutathione peroxidase (PHGPX), an enzyme distinct from the classical glutathione that cannot utilize intact phospholipids.

ATG inhibited, in addition to the classical selenium-dependent glutathione peroxidase, both cytosolic and microsomal (basal and N-ethyl maleimide-stimulated) glutathione S-transferase activities with greater than 80% inhibition achieved at 100 μM ATG. ATG, at concentrations up to 250 μM, did not inhibit PHGPX activity measured by the coupled-enzyme method in the presence of Triton X-100 (0.1%). These data demonstrate the potential of ATG to increase toxicity of lipid peroxidative stimuli by inhibition of microsomal and cytosolic defense mechanisms. Although ATG did not inhibit Triton-enhanced PHGPX activity, overall evidence points toward inhibition of this enzyme as the mechanism for ATG-augmented lipid peroxidation and supports the conclusion that PHGPX plays a major role in the cellular defense mechanism.
Lipids 23, 899–903 (1988).

Several reports indicated that hepatic cytosol contains an unidentified reduced glutathione (GSH)-dependent defense mechanism(s) that protects against microsomal lipid peroxidation (1–5). Because the Se-dependent GSH-peroxidase and GSH-transferase activities of the cytosol are inactive against intact phospholipids (3), their potential involvement in the cytosolic peroxidative defense has been largely discounted. On the other hand, Sevanian et al. (6) have demonstrated that oxidized phospholipids are preferred substrates for phospholipase A_2 (PLA_2) and suggested that PLA_2-mediated release of fatty acyl hydroperoxides would allow GSH-peroxidase and transferase to protect against peroxidative damage, because the enzymes can reduce fatty acyl hydroperoxides (3). In support of this suggestion, we have recently described peroxidation-induced activation of microsomal phospholipase A_1 and A_2 activities (7). In contrast, Gibson et al. (3) have demonstrated that direct addition of partially purified glutathione peroxidase or glutathione S-transferase had no protective effect against lipid peroxidation, and have alternatively described a GSH-dependent cytosolic factor distinct from glutathione peroxidase/transferase activities that prevents the initiation of peroxidation reactions (4). In addition, Ursini et al. (5) have demonstrated a GSH-dependent phospholipid hydroperoxide glutathione (PHGPX) that reduces intact phospholipid hydroperoxides to the corresponding alcohols. This enzyme, thus, potentially could protect against propagation reactions.

In addition to the cytosolic defense against microsomal peroxidation, microsomes, themselves, contain a GSH-dependent microsomal defense mechanism (8). This defense mechanism manifests itself by a lag period, the length of which correlates directly with the microsomal vitamin E content. No particular enzyme involved in this protective effect has been elucidated. One possible candidate for this enzymatic role is microsomal GSH S-transferase, although its activity is much lower than that present in cytosol (9). The ability to greatly augment the microsomal GSH S-transferase by N-ethyl maleimide (10) suggests substantial latent activity of the enzyme. This latent activity of microsomal GSH S-transferase may be responsible for peroxidative protection following the lag phase.

Aurothioglucose (ATG) is a potent inhibitor of the classical cytosolic glutathione peroxidase (11,12). We postulated that this agent could be used to explore further the roles of cytosolic glutathione peroxidase, as well as related GSH-dependent enzymes in lipid peroxidation. The purpose of this study, therefore, was to further define the effects of ATG on microsomal and cytosolic protective enzyme activities and on lipid peroxidation.

EXPERIMENTAL PROCEDURES

Isolation of microsomes and cytosol. Male Sprague-Dawley rats (200-300 g) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Liver microsomes were obtained by differential centrifugation from a 10% homogenate in 0.15 M KCl, 10 μM Tris buffer, pH 7.4. The 100,000 × *g* pellet obtained from the 20,000 × *g* supernatant was washed by recentrifuging at 100,000 × *g*, was resuspended in homogenization buffer (5-10 mg of protein/ml) and assayed for protein content (13). The first 100,000 × *g* supernatant was dialyzed overnight against homogenization buffer and stored frozen at −40 C.

Lipid peroxidation assay. Microsomes (500 μg of protein/ml) were incubated at 37 C in 100 mM Tris buffer, pH 7.,4 (0.5 ml total volume), in the presence of the described peroxidative stimuli and the indicated levels of glutathione, ATG and/or cytosol. Following incubation, the method of Mihara et al. (14) was used to

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Abbreviations: ATG, aurothioglucose; GSH, reduced glutathione; PHGPX, phospholipid hydroperoxide glutathione peroxidase; PLA_2, phospholipase A_2; TBA, 2-thiobarbituric acid.

derivatize 2-thiobarbituric acid (TBA)-reactive substances that were assayed spectrofluorometricaly by the method of Yagi (15).

Preparation of phospholipid hydroperoxides and phospholipid liposomes. Phospholipid hydroperoxides were prepared from egg phosphatidylcholine (Type III E, Sigma Chemical Co., St. Louis, MO) using soybean lipoxygenase-I (Sigma), as described by Eskola and Laakso (16). Chloroform/methanol solutions of phospholipid hydroperoxide and fresh unoxidized phosphatidylcholine were combined such that 10% of the lipid was hydroperoxide. Following evaporation of organic solvents, phospholipid liposomes were prepared by sonication in water to a concentration of 1 mM phosphatidylcholine. Control phospholipid liposomes containing only the fresh phosphatidylcholine were prepared in parallel.

Enzyme assays. Glutathione S-transferase activity was measured with 1-chloro-2,4-dinitrobenzene by the method of Habig et al. (17). Microsomal glutathione S-transferase was activated by N-ethyl maleimide pretreatment, as described by Morgenstern et al. (10). Total glutathione peroxidase activity was measured using a t-butyl hydroperoxide substrate (18). Superoxide dismutase activity was measured by inhibition of superoxide anion-mediated reduction of cytochrome c (19). PHGPX activity was measured by the glutathione peroxidase assay using as substrate phosphatidylcholine hydroperoxide-containing phosphatidylcholine liposomes (prepared as described) at a concentration of 200 μM. Triton X-100 augments PHGPX activity (20) and was required for its measurement (0.1%) final concentration. Controls included activity in the absence of phospholipid or glutathione or using fresh phosphatidylcholine liposomes.

RESULTS

As shown in Figure 1, the peroxidative response of microsomes exposed to ferrous sulfate (10 μM) in the presence of GSH (800 μM) was potentiated by ATG in a dose-dependent manner. However, it did not induce lipid peroxidation in the absence of ferrous ion. In contrast, heat inactivation of microsomes (heated at 100 C for 10 min.) resulted in no increase in lipid peroxidation by any dose (10–200 μM) of ATG. In addition, peroxidation of untreated (not heat inactivated) microsomes in the absence of GSH was not affected by ATG (data not shown). Ferrous ion-induced lipid peroxidation was increased with increased levels of GSH as follows: 100 μM GSH, 210%; 200 μM GSH, 225%; 400 μM GSH, 320%; and 800 μM GSH, 335%. In an effort to observe the effects of ATG on microsomal GSH-dependent protective activity, we subsequently used the ferric ion/ascorbic acid system as a peroxidation stimulus, which is better defined in this regard (8). The peroxidative response of ferric ion/ascorbic acid exhibited a GSH-dependent lag period as previously reported (8). ATG (100 μM) did not affect the length of the lag (obtained using 3 mM GSH), but increased the rate of peroxidation once underway (Fig. 2). Addition of cytosol (25–100 μl from a 10% homogenate/500 μl incubation) inhibited the microsomal peroxidative response to ferric ion/ascorbic acid (measured at 30 min; post lag period). ATG (100 μM) completely reversed the cytosolic inhibition, and 25–50 μM ATG exhibited partial inhibition of the cytosolic protection (Fig. 3).

In order to further explore the effects of ATG on cytosolic protective enzymes, we utilized liposomal sub-

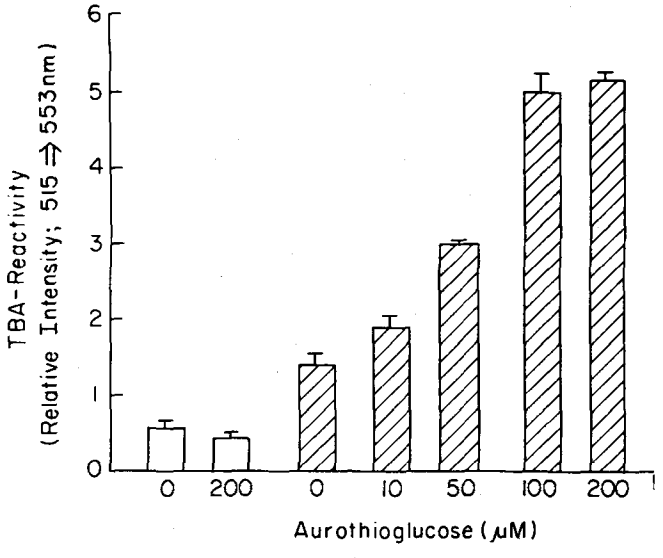

FIG. 1. Promotion of ferrous ion-induced lipid peroxidation by ATG. Rat liver microsomes were exposed to ferrous sulfate (10 μM) (cross-hatched bars) or to control 15 min incubation (open bars) in the presence of the indicated concentration of ATG. All reaction mixtures contained glutathione (800 μM). Lipid peroxidation was assessed by a spectrofluorometric assay of TBA-reactivity as described in the methods. Brackets denote the SEM of 6 observations.

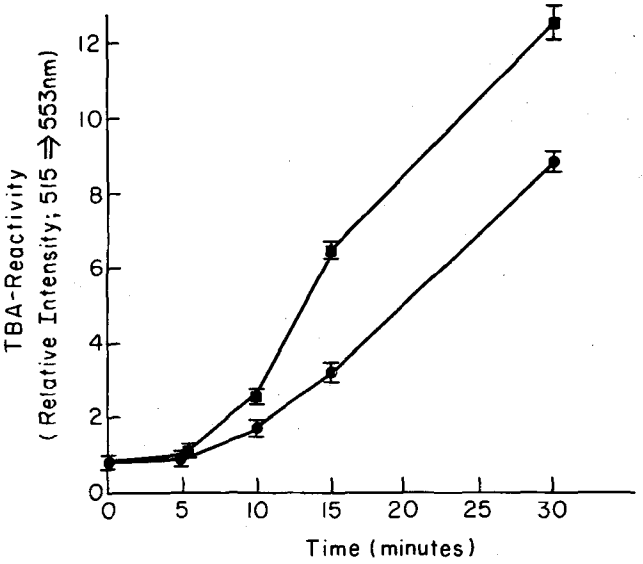

FIG. 2. Potentiation of the postlag phase ferric ion/ascorbic acid-promoted lipid peroxidation by ATG. Rat liver microsomes were exposed to ferric chloride (10 μM) and ascorbic acid (500 μM), in Tris buffer containing 3 mM GSH and no additional agents (closed circles) or ATG (100 μM) (closed squares), for the indicated duration. Lipid peroxidation was assessed as described in Figure 1 and in the methods. Brackets denote the SEM of 4 samples.

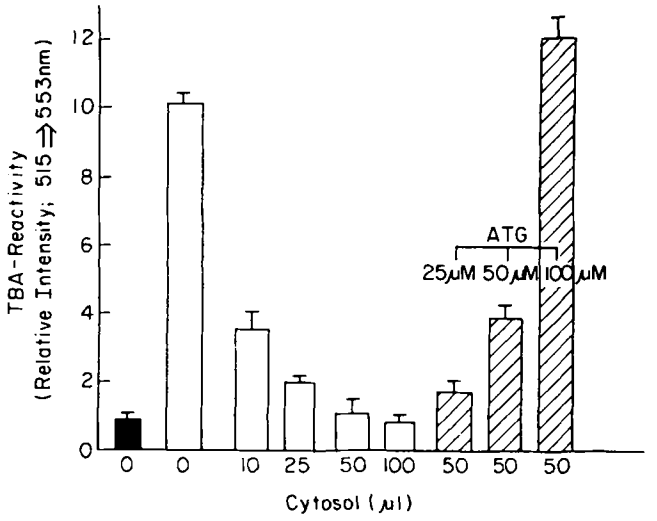

FIG. 3. Inhibition of ferric ion/ascorbic acid-induced lipid peroxidation by cytosol and reversal by ATG. Rat liver microsomes were exposed for 30 min to the peroxidation stimulus ferric chloride (10 μM/ascorbic acid (500 μM), in the presence of GSH (3 mM) and the indicated level of dialyzed cytosol and ATG (cross-hatched bars). Lipid peroxidation was assessed as described above. Brackets denote the SEM of 4 observations.

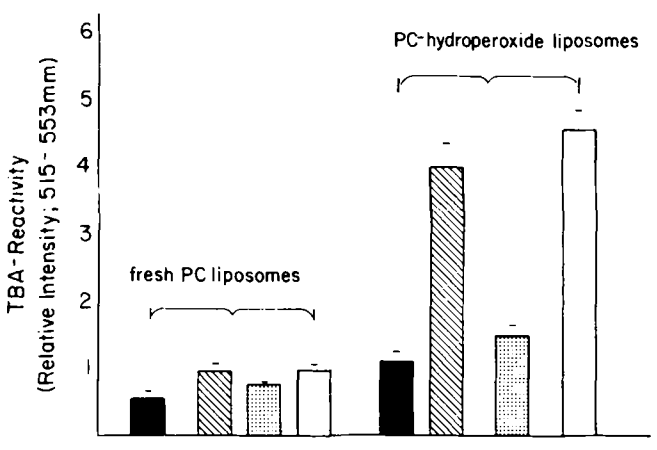

FIG. 4. Inhibition of phosphatidylcholine hydroperoxide-dependent lipid peroxidation by cytosol and reversal by ATG. Rat liver cytosol (or control incubation) was preincubated for 5 min in Tris buffer, pH 7.4, with liposomes prepared from fresh solutions of phosphatidylcholine or liposomes containing both phosphatidylcholine and phosphatidylcholine hydroperoxides (10% of phospholipid oxidized). Some preincubations also contained ATG (100 μM final concentration). Peroxidation reactions were induced by subsequent incubation for 15 min with ferrous sulfate (10 μM), except where noted, and lipid peroxidation assessed as described above. Solid bars; without iron, cross-hatched bars; iron only, dotted bars; cytosol (50 μl) and iron, open bars; cytosol (50 μl) + ATG (100 μM) + iron. Brackets denote the SEM of four observations.

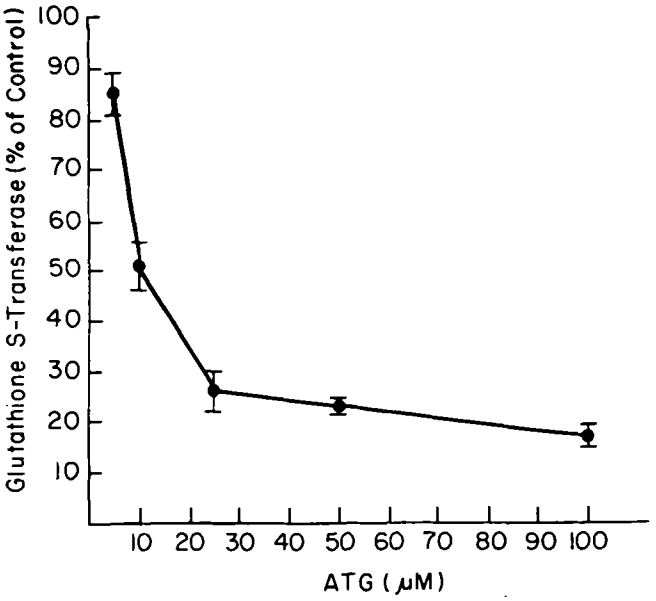

FIG. 5. Inhibition of cytosolic glutathione S-transferase by ATG. Glutathione S-transferase activity was measured as described in the methods. Brackets denote the SEM of 4 observations.

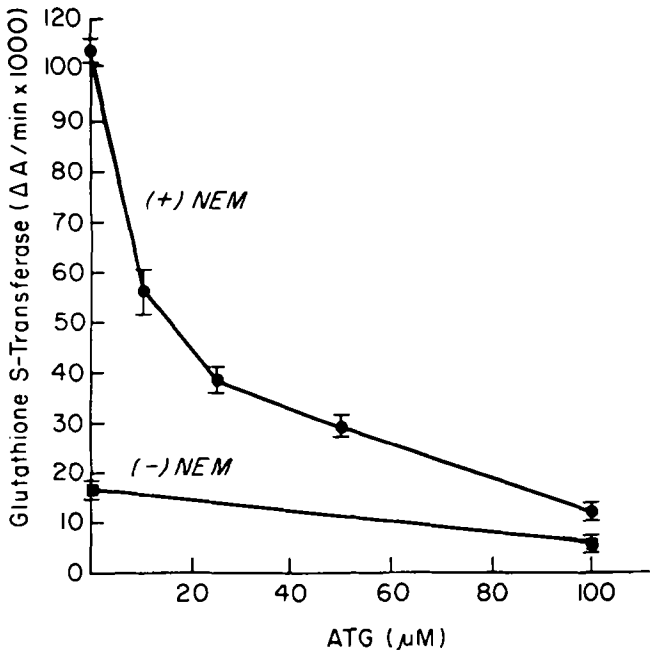

FIG. 6. Inhibition of microsomal glutathione S-transferase activity by ATG. Basal levels of microsomal glutathione S-transferase activity (closed squares) and N-ethyl maleimide augmented activity (closed circles) were measured, as described in the methods, in the presence of the indicated concentration of ATG. Brackets denote the SEM of 4 observations.

strates, consisting of sonicates of phosphatidylcholine containing phosphatidylcholine hydroperoxide (10% of total phospholipid) or of equivalent liposomes prepared from fresh phosphatidylcholine solutions only. Cytosol (with GSH, 500 μM) was preincubated with ATG (5 min), prior to the addition of phospholipid hydroperoxide liposomes and an additional 15 min preincubation. Ferrous sulfate (10 μM) then was added to promote peroxidation. Control experiments were run in the absence of ATG and/or using fresh phosphatidylcholine liposomes. ATG (100 μM final concentration) reversed the inhibition of peroxidation of phosphatidylcholine hydroperoxide liposomes afforded by cytosol (Fig. 4). Liposomes prepared from fresh phosphatidylchyoline (without added hydroperoxide) were peroxidized to a much lesser degree than the hydroperoxide-containing liposomes (Fig. 4),

indicating that most of the peroxidative response in this system resulted from iron-induced phospholipid hydroperoxide-dependent propagation reactions, rather than initiation. Thus ATG, appears to reverse an inhibition of propagation reactions afforded by cytosol.

ATG inhibited cytosolic glutathione S-transferase activity (Fig. 5) and both the basal and the N-ethyl maleimide-augmented microsomal glutathione S-transferase (Fig. 6). The dose-response curves were similar for the ATG-induced inhibition of cytosolic and augmented microsomal activities, with greater than 80% inhibition achieved at 100 μM ATG. Total glutathione peroxidase activity of cytosol, measured using t-butyl hydroperoxide substrate, was inhibited by ATG (72.5% by 100 μM, in close agreement with previous studies [13]. ATG did not affect microsomal or cytosolic superoxide dismutase activity (data not shown). ATG, at concentrations up to 250 μM, did not inhibit PHGPX activity measured in the presence of Triton X-100 (0.1%). PHGPX activity was not detectable in the absence of Triton. (Control experiments showed that Triton, itself, does not elicit apparent activity.)

DISCUSSION

These results have demonstrated that the glutathione peroxidase inhibitor, ATG is a potent enhancer of iron-induced microsomal lipid peroxidation (in the absence of cytosol) and an inhibitor of the cytosolic defense against microsomal lipid peroxidation. The former (microsomal) response to ATG was heat labile (i.e., heat inactivated microsomes were readily peroxidized, with or without ATG, to an equal extent) and was GSH-dependent, suggesting that ATG was inhibiting a GSH-dependent microsomal protein. ATG, however, did not affect the lag period for iron-induced microsomal lipid peroxidation (in the presence of GSH) that has been associated with vitamin E and a GSH-dependent microsomal protein, but instead inhibited peroxidative responses following the lag period. Thus, ATG appears to inhibit a separate GSH-dependent microsomal defense mechanism and, from that, causes the lag period. The cytosolic inhibition was GSH-dependent, as described previously (1-5), and the effect of ATG in overcoming the cytosolic defense was similarly due to inhibition of a cytosolic GSH-dependent enzyme. It is unlikely that glutathione depletion is a major mechanism for the peroxidative response to ATG, as the potentiation was higher using a 800 μM level of GSH than at 100-200 μM GSH.

ATG reversed phospholipid hydroperoxide-dependent liposomal lipid peroxidation (propagation) promoted by iron. This effect could not be due to the classical glutathione peroxidase or glutathione S-transferase, as these enzymes are inactive against intact phospholipids (3), and phospholipases are absent in this system. The activity reported by Gibson et al. (4) inhibits initiation and, thus, should not be involved in a phospholipid hydroperoxide-dependent response. The PHGPX described by Ursini (5) utilizes the phospholipid hydroperoxide substrate and likely is responsible for the cytosolic inhibition that is blocked by ATG. ATG, however, did not inhibit PHGPX using the coupled enzyme assay with NADPH-dependent glutathione reductase and Triton X-100. Measurement of PHGPX by this assay is difficult, using crude enzyme sources (5), but is facilitated by the addition of Triton X-100 (20). It is possible that ATG effects might be masked under the conditions appropriate for this assay.

The possible effect of ATG on cytosolic PHGPX does not explain why microsomal lipid peroxidation was enhanced in the absence of cytosol, and raises the possibility that the reversal of cytosolic inhibition was due to a stimulatory mechanism. The GSH-dependence and heat-labile nature of the effect of ATG on iron-induced microsomal peroxidation argue for the involvement of GSH-dependent enzymes. Although largely recovered in the soluble portion of tissues, PHGPX has been extracted from microsomes and mitochondria using high ionic strength treatment (21), and thus may be involved in the in vitro microsomal peroxidation system. ATG did not enhance liposomal lipid peroxidation in the absence of PC-hydroperoxide and cytosolic enzymes (Fig. 4), indicating that nonenzyme-related stimulatory mechanisms probably are not operative with microsomes.

ATG inhibited GSH S-transferase activity, in addition to GSH peroxidase activity. Although GSH S-transferase apparently is not involved in protection against lipid peroxidation (3), as assessed by general determinations, it is possible that the role of this enzyme is glutathione conjugation reactions may affect the overall toxicity of a peroxidative response. In this regard, the bioactive aldehydic lipid peroxidation product 4-hydroxy nonenal has recently been reported to be a substrate for glutathione S-transferase (conjugation reaction) (22). Investigations of levels of specific peroxidation products may better define the importance of this and similar reactions of glutathione S-transferase.

In conclusion, our results have demonstrated that ATG inhibits cytosolic and microsomal defense mechanisms against lipid peroxidation. In view of the multiple effects of the agent on enzyme activities, it it not possible to draw absolute conclusions about the protective enzyme associated with ATG-induced peroxidative responses. The reversal of the cytosolic inhibition of phospholipid hydroperoxide-dependent propagation reactions by ATG, however, does suggest strongly an involvement of PHGPX, despite the lack of effect of ATG on the associated enzyme assay. The implication of a prominent role for PHGPX in the peroxidation defense mechanism argues against the suggested role of phospholipases A as a protective agents (6).

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REFERENCES

1. McCay, P.B., Gibson, D.D., Fong, K.L., and Hornbrook, K.R. (1976) *Biochim. Biophys. Acta 431*, 459-468.
2. Burk, R.F., Trumble, M.J., and Lawrence, R.A. (1980) *Biochim. Biophys. Acta 618*, 35-41.
3. Gibson, D.D., Hornbrook, K.R., and McCay, P.B. (1980) *Biochim. Biophys. Acta 620*, 572-582.
4. Gibson, D.D., Hawrylko, J., and McCay, P.B. (1985) *Lipids 10*, 704-711.

5. Ursini, F., Valente, M.M., Ferri, L., and Gregolin, C. (1982) *Biochim. Biophys. Acta 710,* 197-211.
6. Sevanian, A., Muakkassa-Kelly, S.F., and Montresque, S. (1983) *Arch. Biochem. Biophys. 223,* 441-452.
7. Beckman, J.K., Borowitz, S.M., and Burr, I.M. (1987) *J. Biol. Chem. 262,* 1479-1481.
8. Hill, K.E., and Burk, R.F. (1984) *Biochem. Pharmacol. 33,* 1065-1068.
9. Glatt, H., and Oesch, F. (1977) *Arch. toxicol 39,* 87-96.
10. Morgenstern, R., DePierre, J.W., and Ernster, L. (1979) *Biochem. Biophys. Res. Commun. 87,* 657-663.
11. Mercurio, S.D., and Combs, G.F. (1985) *J. Nutr. 115,* 1459-1470.
12. Chaudiere, J., and Tappel, A.L. (1984) *J. Inorg. Biochem. 20,* 313-325.
13. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-285.
14. Mihara, M., Uchiyama, M., and Fukuzawa, K. (1980) *Biochem. Med. 23,* 302-311.
15. Yagi, K. (1984) in *Methods of Enzymology* (Packer, L., ed.) Vol. 105, pp. 328-331, Academic Press, New York, NY.
16. Eskola, J., and Laakso, S. (1983) *Biochim. Biophys. Acta 751,* 305-311.
17. Habig, W.H., Pabst, M.J., and Jacoby, W.B. (1974) *J. Biol. Chem. 249* 7130-7139.
18. Paglia, D.E., and Valentine, W.N. (1967) *J. Lab. Clin. Med. 70,* 158-169.
19. McCord, J.M., and Fridovitch, I. (1969) *J. Biol. Chem. 244,* 6049-6055.
20. Maiorino, M., Roveri, A., Gregolin, C., and Ursini, F. (1986) *Arch. Biochem. Biophys. 251,* 600-605.
21. Ursini, F. and Bindoli, A. (1987) *Chem. Phys. Lipids 45,* 255-275.
22. Alin, P., Danielson, H., and Mannervik, B. (1985) *FEBS Lett. 179,* 267-270.

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Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) on de novo Fatty Acid and Cholesterol Synthesis in the Rat

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The effects of 1, 5, 10 and 20 μg/kg dosages of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) upon de novo fatty acid and cholesterol synthesis in liver and adipose tissue were determined in pair-fed rats. The incorporation of tritium from ^3H_2O into tissue lipids was measured. Hepatic and adipose fatty acid synthetic rates (μmoles acetyl units g^{-1} hr^{-1}) in the control groups were 19.6 ± 4 and 75.7 ± 18.5, respectively, and the liver cholesterol synthetic rate was 2.9 ± 0.5. TCDD (1 μg/kg) inhibited fatty acid synthesis in the liver and adipose tissue, by 44% and 41%, respectively, and the liver cholesterol synthesis was inhibited by 37%. The extent of these inhibitions increased with increasing dosages of TCDD. The effect of TCDD on sterol synthesis in adipose tissue could not be determined, because the tritium incorporation into the sterol fraction in this tissue was not detectable.
***Lipids 23,* 904–906 (1988).**

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic contaminant of the herbicides used as defoliants. It also has been implicated in several accidental poisonings (1–3). So far, the mechanism of its toxicity (acute or chronic) has not been elucidated. Our recent studies on long-term pharmacokinetics of TCDD (4) have established that orally administered TCDD is transported in the blood compartment as chylomicrons and that the bulk of it is deposited in the liver and adipose tissues with half-lives of 5.3 and 7.6 weeks, respectively. Therefore, these two tissues seem to be the major target organs for the toxic manifestations of TCDD.

TCDD dramatically alters the lipid profiles in laboratory animals and man. Elegant studies by Albro et al. (5) and McConnell et al. (6) have shown clearly that a sublethal dose of TCDD causes a transient increase in serum triglycerides and free fatty acids and a decrease in steryl esters, while a lethal dose causes fatty liver and large increases in serum cholesteryl esters and free fatty acids without significant change in triglyceride levels. Attempts to determine the effects of TCDD on hepatic lipid synthesis have been inconclusive, because of the use of labeled acetate as the precursor for the measurement of absolute rates of lipid synthesis (7). Since the concentration of the intracellular pool of acetate can vary significantly, depending on the nutritional state of the animal, the specific activity of injected labeled acetate in vivo cannot be accurately determined. Therefore, measurements of lipid synthetic rates based on labeled acetate incorporation have been questioned (8–11). In contrast, the incorporation of tritium from tritiated water into body lipids has been demonstrated to give an accurate measure of lipid synthetic rates because the specific activity of body water can be determined precisely (8–11). Furthermore, because the lipid synthetic rates are maximal in fully fed animals, it is important to perform these studies under carefully controlled conditions, such as in a meal-fed state (12,13). As shown by Seefeld and Peterson (14) and by Gorski and Rozman (15), TCDD administration significantly affects the food consumption for at least 5 days. In order to rule out any secondary effects of TCDD on lipid synthesis due to impaired food consumption, the control and experimental animals were not only meal-fed, but also pair-fed, for seven days in the present studies so that the true effects of TCDD on hepatic and adipose tissue synthetic rates of de novo fatty acid and cholesterol could be determined. It will be demonstrated that a dosage of TCDD as low as 1 μg/kg causes a significant inhibition of both fatty acid and cholesterol synthesis in the liver and of fatty acid synthesis in the adipose tissue.

MATERIAL AND METHODS

TCDD. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was purchased from the IIT Research Institute Chemical Carcinogen Standard Reference Repository, Chicago, IL, and was found to be more than 98% pure, based on high-performance liquid chromatography (HPLC) (4). All other chemicals and reagents were of analytical grade.

Animals. Wistar rats (body wt 170–190 g) were procured from Charles River, Wilmington, MA, and were maintained on the normal Wayne Lablox chow (4.4% fat content) ad libitum for two weeks before experimentation. All the rats were meal-fed the same diet between 9 am and 12 noon every day for a week and divided into control and experimental groups of at least six rats each.

Effect of dosage of TCDD. Each animal in the experimental groups received an intraperitoneal injection of 1, 5, 10 or 20 μg/kg body wt of TCDD in corn oil. The animals in the corresponding control groups received an equivalent volume of corn oil vehicle only. All the animals were meal-fed for a further period of one wk. The animals in any control group were pair-fed with the animals in the corresponding experimental group.

Determination of fatty acid and cholesterol synthetic rates in liver and adipose tissues in vivo. The tritium incorporation method is based on the work of Fain and Scow (8), as well as that of Jungus (9), and has been successfully validated by Brunengraber et al. (10) and by Lakshman and Veech (11). Briefly, the rats from all the groups were injected intraperitoneally with tritiated water (1 mCi/kg body wt) at the end of the last meal-feeding period. They were killed after 60 min and the blood plasma, liver and the adipose tissue were saved, and the lipid synthetic rates were measured as described previously (10–13).

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Abbreviations: HPLC, high-performance liquid chromatography; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD.

RESULTS

Effect of various dosages of TCDD on the weights of body, liver and fat pads. The effect of TCDD administration at dosages of 1, 5, 10 and 20 μg/kg body wt on the weights of body, liver and fat pads are presented in Table 1. TCDD at the dosages of 1, 5 and 20 μg/kg did not significantly affect the body weight within seven days of treatment. However, the mean body weight of the animals receiving 10 μg/kg TCDD was significantly lower than that of the animals in the corresponding control group ($P < 0.01$). Long-term treatment of rats with TCDD, especially at higher dosages, is known to reduce the body weight (14,15). In contrast, the mean liver weight increased with increasing dosage of TCDD. Thus, the liver weight expressed as percentage of the body weight was 15% higher at 1 μg/kg and 46% higher at 20 μg/kg dosage ($P < 0.01$), compared with the corresponding control groups. Significantly, TCDD failed to affect the weight of the epididymal fat pads at least at the dosages and duration of exposure in the present studies (Table 1).

Effect of varying dosages of TCDD on de novo hepatic fatty acid and cholesterol synthesis. Hepatic de novo fatty acid and cholesterol synthetic rates in TCDD-treated and pair-fed control animals are reported in Table 2. The hepatic fatty acid synthetic rate was 19.6 μmol acetyl units incorporated per gram per hour, and the cholesterol synthetic rate was 2.9 μmol acetyl units incorporated per gram per hour, in the control animals corresponding to the 1 and 5 μg/kg TCDD groups. The fatty acid synthetic rate in the adipose tissue of control animals of these groups was 75.7 μmol acetyl units incorporated per gram per hour. TCDD, at 1 and 5 μg/kg dosages, caused significant inhibitions of hepatic fatty acid and cholesterol syntheses (Table 2). These inhibitory effects increased with increasing dosage of TCDD, in spite of the fact that the synthetic rates in the control animals pair-fed with the animals in 10 and 20 μg/kg experimental groups were significantly lower than the corresponding rates observed in the control animals pair-fed with the animals from 1 and 5 μg/kg experimental groups (Table 2). The tritium incorporation into nonsaponifiable fraction also was inhibited proportionately with increasing dosage of TCDD (data not shown).

Adipose tissue also showed a marked 41% inhibition of fatty acid synthesis at a dosage of 1 μg/kg TCDD (Table 2). The extent of this inhibition increased gradually with increasing dosage of TCDD. The effect of TCDD on sterol synthesis in adipose tissue could not be determined

TABLE 1

Effect of Various Dosages of TCDD On the Weights of Body, Liver and Fat Pads

	Dosage μg/kg	# of animals	Body wt		Liver wt		Fat pad wt	
			Initial	Final	g	As % of body wt	g	As % of body wt
Control		6	195 ± 9	220 ± 3	6.5 ± 0.2	2.96	2.1 ± 0.1	0.88
Exptl	1.0	6	189 ± 9[a]	211 ± 4[a]	7.1 ± 0.2	3.39[b]	2.0 ± 0.2	0.86[a]
Control		6	195 ± 9	220 ± 6	6.5 ± 0.2	2.96	2.1 ± 0.1	0.88
Exptl	5.0	6	188 ± 9[a]	210 ± 8[a]	7.6 ± 0.2	3.7[b]	2.0 ± 0.07	0.89[a]
Control		6	190 ± 7	206 ± 6	6.0 ± 0.2	2.91	1.6 ± 0.09	0.79
Exptl	10.0	9	186 ± 9[a]	191 ± 8[b]	7.9 ± 0.5	4.13[b]	1.6 ± 0.16	0.86[a]
Control		6	191 ± 5	220 ± 6	6.5 ± 0.4	2.96	1.7 ± 0.15	0.80
Exptl	20.0	10	197 ± 7	212 ± 9[a]	9.2 ± 0.4	4.33[b]	1.6 ± 0.13	0.76[a]

Each value is the mean ± SD.

[a]Not significant compared with the corresponding control group.

[b]$P < 0.01$ compared with the corresponding control group.

TABLE 2

Effect of Various Dosages of TCDD On Lipid Synthesis

Tissue	Lipid class	Lipid synthetic rate[a] in control animals		% Inhibition at TCDD dosages of μg/kg			
		1 & 5 μg group	10 & 20 μg group	1	5	10	20
Liver	Fatty acids	19.6 ± 4.0	12.6 ± 3.0	44	48	52	62
	Cholesterol	2.96 ± 1.2	1.7 ± 0.8	37	42	52	63
Adipose	Fatty acids	75.7 ± 18.5	38.0 ± 14.4	41	44	54	56

Various groups of rats were injected with the indicated dosages of TCDD and meal-fed for 7 days. The corresponding groups of control animals received the corn oil vehicle only and were pair-fed with the respective experimental groups. Lipid synthetic rates in the liver and adipose tissues were determined as described in "Materials and Methods" section. The observed inhibitions in the experimental groups are reported as % of the rates observed in the corresponding control groups.

[a]μmoles acetyl units g^{-1} hr^{-1}; each value is the mean ± SD.

because the tritium incorporation into sterol fraction in this tissue was not detectable.

DISCUSSION

It is well known that TCDD can cause significant weight loss and depletion of body fat, depending on its dosage and the duration of exposure (5,16-20). In the present studies, TCDD was found to affect significantly the body weight of the animals only at the dosage of 10 μg/kg, seven days after exposure (Table 1). This may have been due to slightly lower initial body weight of this group compared with its corresponding control group. Furthermore, none of the dosages of TCDD used in this study affected the epididymal fat pad weight. In contrast, the liver weight increased markedly at all the dosages of TCDD tested, presumably due to hypertrophy or hyperplasia. In fact, the liver weight, as percentage of the body weight, increased by 46% ($p < 0.01$) in the 20 μg/kg experimental group compared with the corresponding control group (Table 2).

The observed rates of fatty acid and cholesterol syntheses in the control animals pair-fed with the animals in the 1 and 5 μg/kg experimental groups (Table 2) are comparable with physiological rates (12,13) found in normal meal-fed rats. The significantly lower lipid synthetic rates observed in the control group pair-fed with the 10 and 20 μg/kg experimental group likely are due to the fact that pair-feeding led to a lower amount of food consumed by the control group, corresponding to higher dosages of TCDD (8 ± 1 g/day) than those corresponding to lower dosages of TCDD (13 ± 1.2 g/day). Nonetheless, TCDD inhibited markedly the absolute rates of both fatty acid and cholesterol synthesis in the liver and fatty acid synthesis in the adipose tissue, even at a dose of 1 μg/kg body wt (Table 2). The extent of this inhibition increased with increasing dosage of TCDD. The reason the magnitude of this inhibition did not become more severe at 10 and 20 μg/kg TCDD dosages may be due to the fact that the lipid synthetic rates in these control groups themselves were lower than the rates observed in the control groups corresponding to lower dosages (Table 2).

It must be pointed out that the absolute rate of lipid synthesis calculated on the basis of tritium incorporation is dependent on the constant amount of $^3H/C$ ratio, as reported by a number of workers (8-11,21-23). However, this has been questioned by in vitro studies (24,25) in isolated hepatocytes, where this ratio was shown to vary with the time of incubation and by the addition of pyruvate and glucagon to the incubation medium. But, the pioneering work of Dietschy and his associates (21-23) indicates clearly that the $^3H/C$ ratio remains constant for each molecule of sterol, synthesized in both liver and intestine, that is independent of the metabolic state, the rate of lipid synthesis and the type and concentration of the substrate from which the acetyl CoA pool is derived. Therefore, the tritium incorporation method is the most accurate of currently available methods for measuring the absolute rate of lipid synthesis. Whether TCDD treatment affects the $^3H/C$ ratio remains to be clarified.

Based on the present data, it is reasonable to conclude that, long before its toxic manifestations on body weight and depot fat, TCDD impairs body lipid synthetic rates within a week at as low as 1 μg/kg dosage (equivalent to endogenous TCDD concentration of 1 ppb). This biochemical effect may have important clinical implications in TCDD induced weight and fat loss in human populations who might have accumulated as low as 1 ppb TCDD after exposure. Further studies on the duration and mechanism of action of TCDD on lipid synthetic rates are currently in progress.

REFERENCES

1. Kimbrough, R.D. (1974) *CRC Crit. Rev. Toxicol. 2,* 445-498.
2. Poland, A., and Kende, A. (1976) *Fed. Proc. 35,* 2404-2411.
3. Reggiani, G. (1978) *Arch. Toxicol. 40,* 161-187.
4. Lakshman, M.R., Campbell, B.S., Chirtel, S.J., Ekarohita, N., and Ezekiel, M. (1986) *J. Pharm. Exp. Ther. 239,* 673-677.
5. Albro, P.W., Corbett, J.T., Harris, M., and Lawson, L.D. (1978) *Chem. Biol. Interact. 23,* 315-330.
6. McConnell, E.E., More, J.A., and Dalgaard, D.W. (1978) *Toxicol. Appl. Pharmacol. 43,* 175-187.
7. Cunningham, H.M., and Williams, D.T. (1972) *Bull. Environ. Contam. Toxicol. 7,* 45-51.
8. Fain, J.N., and Scow, R.O. (1966) *Am. J. Physiol. 210,* 19-25.
9. Jungus, R.L. (1968) *Biochemistry 7,* 3708-3717.
10. Brunengraber, H., Sabine, J., Boutry, R.M., and Lowenstein, J.M. (1972) *Arch. Biochem. Biophys. 150,* 392-396.
11. Lakshman, M.R., and Veech, R.L. (1977) *J. Biol. Chem. 252,* 4667-4673.
12. Lakshman, M.R., Muesing, R.A., Cook, G.A., and Veech, R.L. (1977) *J. Biol. Chem. 252,* 6581-6583.
13. Lakshman, M.R., Berdanier, C.D., and Veech, R.L. (1977) *Arch. Biochem. Biophys. 183,* 355-360.
14. Seefeld, M.D., and Peterson, R.E. (1983) in *Human and Environmental Risks of Chlorinated Dioxins and Related Compounds* (Tucker, R.E., Young, A.L., and Gray, A.P., eds.) pp. 406-413, Plenum Press, New York, NY.
15. Gorski, J.R., and Rozman, K. (1987) *Toxicology 44,* 297-307.
16. Harris, M.W., Moore, J.A., Vos, J.G., and Gupta, B.N. (1973) *Environ. Health Perspect. 5,* 101-109.
17. Vos, J.G., Moore, J.A., and Zinkl, J.G. (1974) *Toxicol. Appl. Pharmacol. 29,* 229-241.
18. McConnell, E.E., Moore, J.A., Haseman, J.K., and Harris, M.W. (1978) *Toxicol. Appl. Pharmacol. 44,* 335-356.
19. McNulty, W.P. (1977) *Bull. Environ. Contam. Toxicol. 18,* 108-109.
20. Kociba, R.J., Keeler, P.A., Park, C.N., and Gehring, P.J. (1976) *Toxicol. Appl. Pharmacol. 35,* 553-574.
21. Andersen, J.M., and Dietschy, J.M. (1979) *J. Lipid Res. 20,* 740-752.
22. Stange, E.F., and Dietschy, J.M. (1983) *J. Lipid Res. 24,* 72-82.
23. Dietschy, J.M., and Spady, D.K. (1984) *J. Lipid Res. 25,* 1469-1476.
24. Pullinger, C.R., and Gibbons, G.F. (1983) *J. Lipid Res. 24,* 1321-1328.
25. Bjornsson, O.G., Pullinger, C.R., and Gibbons, G.F. (1985) *FEBS Lett. 187,* 302-306.

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Genetic Basis for Obesity and Diabetes (October 30–November 1, 1988, Stouffer Pineisle Resort, Lake Lanier Islands, Georgia). Sponsored by the University of Georgia, Center for Continuing Education and the College of Home Economics. For more information, contact Dr. Carolyn D. Berdanier, Conference Chair, University of Georgia, Department of Foods and Nutrition, Dawson Hall, Athens, GA 30602.

Vitamin E: Biochemistry and Health Implications (October 31–November 3, 1988, The Sheraton Centre, New York, NY). Sponsored by The New York Academy of Sciences. For more information, contact the Conference Department, The New York Academy of Sciences, 2 East 63rd St., New York, NY 10021, (212) 838-0230.

International Symposium on Clinical, Biochemical and Molecular Aspects of Fatty Acid Oxidation (November 6–9, 1988, Penn Tower Hotel, Philadelphia, PA). For more information, contact Paul M. Coates, Ph.D., Division of Genetics, The Children's Hospital of Philadelphia, 34th St. and Civic Center Blvd., Philadelphia, PA 19104.

23rd Annual Hugh Lofland Conference on Arterial Wall Metabolism (May 24–27, 1989, Welches, Oregon). Requests for information should be sent to M. R. Malinow, M.D., Chairman, Hugh Lofland Conference, Oregon Regional Primate Research Center, 505 NW 185th Avenue, Beaverton, OR 97006, (503) 690-5258.

10th Anniversary Meeting of the European Association for Cancer Research (University College, Galway, Ireland, September 11–13, 1989). For further information, contact Dr. S. M. Lavelle, Experimental Medicine, University College, Galway, Ireland; or in North America, contact Dr. J. H. Weisburger, American Health Foundation, Valhalla, New York, NY 10595-1599. The program involves plenary lectures, workshops, symposia and poster sessions.

Lipid Composition of Platelets From Patients With Atherosclerosis: Effect of Purified Eicosapentaenoic Acid Ethyl Ester Administration

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Although eicosapentaenoic acid (EPA) has been shown to have beneficial effects in the prevention of atherosclerosis, the mechanism by which these effects occur is not entirely clear. We investigated the lipid composition of platelets in paired subjects with and without atherosclerotic disease, either hypercholesterolemic (low density lipoprotein [LDL] cholesterol [Chol] ⩾ 170 mg/dl) or normocholesterolemic (LDL-Chol < 170 mg/dl). Platelets from patients with atherosclerotic disease had a lower phosphatidylcholine (PC)/Chol ratio, when compared with those from patients without atherosclerotic disease, irrespective of LDL-Chol levels. Eleven patients with atherosclerotic disease were treated with purified EPA ethyl ester (1.8 g/day), and changes in lipid composition of platelets were investigated. Plasma levels of total Chol and LDL-Chol decreased significantly after EPA administration. The phospholipid (PL)/Chol ratio and the PC/Chol ratio in platelets from patients with atherosclerotic disease increased significantly after 4–10 wk EPA treatment. The EPA content in platelets increased, while the arachidonic acid (AA) content decreased. EPA-induced changes in the PL/Chol and the PC/Chol ratios of platelets, as well as fatty acyl chain shifts, may be related to the beneficial effects in preventing atherosclerosis, possibly by increase in the membrane fluidity. *Lipids 23,* 917–923 (1988).

Platelet activation is thought to be important in the generation of atherosclerotic disease (1). The lipid composition of the platelet affects not only prostaglandin metabolism (2,3), but also membrane fluidity of platelets (4), and thus, might affect platelet aggregation. Determinants of membrane fluidity are the phospholipid (PL)/cholesterol (Chol) ratio and the class of PL, as well as the fatty acid composition in the membrane (5).

Considerable evidence suggests that hypercholesterolemia, particularly the increase in the low density lipoprotein (LDL) Chol fraction, is associated with increased platelet aggregation (6). Patients with atherosclerotic disease also are reported to have enhanced platelet activity (7,8). One of the aims of the present study is to analyze separately the effects of hypercholesterolemia and atherosclerotic disease on the lipid composition of platelets, and to clarify the characteristics of lipid composition including PL class composition in platelets of patients with atherosclerotic disease.

Since Dyerberg et al. (9,10) reported the relationship between the diet of Eskimos and the low incidence of myocardial infarction, which might be accounted for by a reduction in platelet aggregation, the effects of fish diet or a supplement of fish oil on platelet aggregation and prostaglandin metabolism in platelets has been extensively investigated (8,11,12). However, there have been few studies investigating the effect of eicosapentaenoic acid (EPA) administration on the composition of platelet lipids other than fatty acids (13,14). Therefore, in this study, we also investigated the effect of purified EPA ethyl ester on platelet lipid composition, including Chol content and PL class composition, as well as fatty acid composition, in patients with atherosclerotic disease.

MATERIALS AND METHODS

Subjects. Forty-four persons were enrolled in the present study, consisting of 11 subjects with normal LDL-Chol level and without atherosclerotic disease (aged 25–66 yr), 11 with normal LDL-Chol level and with atherosclerotic disease (31–70 yr), 11 with high LDL-Chol level and without atherosclerotic disease (22–68 yr) and 11 with high LDL-Chol level and with atherosclerotic disease (33–63 yr). The clinical findings of each group are shown in Table 1. Patients with atherosclerotic disease included 18 patients with ischemic heart disease, as diagnosed by exercise electrocardiography (six were angiographically confirmed), two with old cerebral infarction as diagnosed by brain computerized tomography and two with peripheral arterial disease as determined by angiography. Plasma LDL-Chol level was calculated by Friedewald's equation: LDL-Chol = total Chol − ([triglycerides/5] + HDL-Chol) (15). These subjects were arbitrarily classified into those with normal plasma LDL-Chol level (<170 mg/dl) and those with high plasma LDL-Chol level (⩾170 mg/dl). The 170 mg/dl plasma level of LDL-Chol was chosen as the "cut off" point, because the level represents the moderate-risk level even in subjects older than 50 (16).

EPA administration. Among the 22 patients with atherosclerotic disease, 11 patients (seven with normal LDL-Chol levels, four with high LDL-Chol levels) were treated with EPA ethyl ester on an outpatient basis. They were selected for their willingness to be treated with EPA ethyl ester. Their clinical characteristics are shown in Table 2. Diet and other drugs administrated concomitantly were unchanged during this study. Subjects were consuming an ordinary Japanese diet, and none were obese. Patient 10 was taking cholestyramine; patient 9, niceritrol (a derivative of nicotinic acid); and the remainder were not taking hypolipidemic medications. Each patient was treated with EPA ethyl ester 1.8 g/day (six capsules/day) in three divided doses for 4–10 wk. Seven patients were studied at four wk. One patient was studied at six wk (patient 4), two at eight wk (patients 5 and 6), and one was studied at 10 wk (patient 3). The encapsulated EPA ethyl ester was provided by Mochida Pharmaceutical Company. Each gelatin-coated soft capsule weighed 400 mg and contained ethyl esters containing 75% EPA, 1–4% C18:4, 1–4% C20:4, 1% C22:5, 4–6%

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Abbreviations: AA, arachidonic acid; Chol, cholesterol; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EDTA, ethylenediamine tetraacetic acid; EPA, eicosapentaenoic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; prot, protein; PS, phosphatidylserine; SM, sphingomyelin; TG, triglyceride(s); GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

TABLE 1

Clinical Findings in Subjects with and without Atherosclerotic Disease, with and without High Plasma Low Density Lipoprotein (LDL)-Cholesterol (≥170 mg/dl)

	LDL-cholesterol <170 mg/dl		LDL-cholesterol ≥170 mg/dl	
	Atherosclerotic disease		Atherosclerotic disease	
	−	+	−	+
Number	11	11	11	11
Atherosclerotic disease				
Ischemic heart disease	0	9	0	9
Old cerebral infarction	9	1	0	1
Peripheral arterial disease	0	1	0	1
Male:Female	7:4	8:3	9:2	6:5
Age[a]	44 ± 16	55 ± 12	49 ± 12	51 ± 9
Plasma lipids[a]				
Cholesterol (mg/dl)	196 ± 36	209 ± 16	308 ± 36	300 ± 53
Triglycerides (mg/dl)	132 ± 57	149 ± 64	131 ± 40	147 ± 56
HDL-Cholesterol (mg/dl)	39 ± 10	48 ± 11	49 ± 13	45 ± 15
LDL-Cholesterol (mg/dl)	131 ± 29	131 ± 18	232 ± 42	228 ± 58

[a]Values are presented as mean ± SD.

TABLE 2

Clinical Findings in Patients with Atherosclerotic Disease before Treatment with Purified Eicosapentaenoic Acid Ethyl Ester (EPA-E)

Patients	Sex[a] age (yrs)	Weight (kg)	Relative[b] weight (%)	Plasma[c] Chol (mg/dl)	Plasma[c] TG (mg/dl)	Plasma[c] HDL-Chol (mg/dl)	Plasma[d] LDL-Chol (mg/dl)	Atherosclerotic[e] disease
1	M 55	54	103	190	148	51	109	IHD
2	M 58	60	106	206	108	64	120	IHD
3	M 70	61	104	227	231	32	149	IHD
4	M 31	64	106	217	143	37	151	PAD
5	M 53	59	104	239	162	50	157	OCI
6	F 61	49	107	191	61	70	109	IHD
7	F 64	41	93	200	121	51	125	IHD
8	M 47	53	100	242	79	52	174	IHD
9	M 53	57	90	300	181	35	229	IHD
10	F 54	54	109	308	174	51	222	IHD
11	F 63	47	100	310	63	85	212	IHD

[a]M, male; F, female.

[b]Calculated as 100 × wt (kg)/[(ht(cm) − 100) × 0.9].

[c]Chol, cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein.

[d]LDL-Chol was calculated as Chol − (TG/5 + HDL-Chol).

[e]IHD, ischemic heart disease; PAD, peripheral arterial disease; OCI, old cerebral infarction.

C22:6 and 0.2% α-tocopherol. Informed consent was obtained from each patient before EPA ethyl ester administration.

Preparation of platelet suspensions. Fresh venous blood was drawn from patients and anticoagulated by collecting it in a plastic tube with EDTA 3.5 mM (final concentration). Platelet-rich plasma was prepared by centrifugation of whole blood at 250 *g* for 10 min. The platelets were then sedimented at 1100 *g* for 10 min and routinely washed two times in a washing buffer (9 mM EDTA phosphate buffered saline, pH 6.2). Microscopic observation revealed that the contamination by erythrocytes was negligible.

Homogenization of the platelets. Isolated platelets were homogenized by sonication with microtip, eight times for 15 seconds at 70% intensity, with intervening cooling periods between them. Platelet protein content was measured by the method of Lowry (17).

Lipid analysis. Lipid extraction of the homogenized platelets was carried out by the method of Folch et al. (18), using a chloroform/methanol solution (2:1, v/v). Platelet Chol content was determined by the method of Rudel and Morris (19). Platelet lipid phosphorus content was estimated by a modified Chen's method (20). Total PL content was taken to equal lipid phosphorus times 25. Plasma Chol and triglyceride (TG) concentrations were

measured by the enzymatic method (21,22). High density lipoprotein (HDL)-Chol levels were evaluated by selective precipitation (heparin-calcium method) (23). Analysis of PL composition was carried out by two-dimensional thin-layer chromatography (TLC) by the method of Rouser et al. (24). Total lipids (approximately 150 μg of PL) were applied to Silica Gel 60 H impregnated with 0.01 M magnesium acetate plate and developed in the first dimension with chloroform/methanol/aq NH_3 (28%)/water, 120:80:10:5 (v/v/v/v) and in the second dimension with chloroform/acetone/methanol/acetate/water, 100:40:30:20:12 (v/v/v/v/v). After visualization of the lipids by charring at 180°C for 30 min with 20% aqueous solution of ammonium sulfate containing 4% H_2SO_4, the silica areas were scraped into tubes and lipid phosphorus was estimated by the method of Rouser et al. (24).

The fatty acids of total lipids in platelets were analyzed as their methyl esters by gas-liquid chromatography (GLC) using a Shimadzu GC-8A gas chromatograph equipped with a hydrogen flame ionization detector. A 2.6 mm i.d. × 2 m glass column packed with SILAR-10C on 100–120 mesh was used. Analyses were carried out at an injection temperature of 250°C and a column temperature of 185°C. The signals were analyzed by a Chromatopac C-R1A (Shimadzu) computer analyzer. Each peak was identified by comparing it with those of assays on standard mixtures of fatty acids supplied by Applied Science Laboratories Inc. A standard mixture including docosapentaenoic acid (DPA) (ω-3) was supplied by Mochida Pharmaceutical Company.

Statistical evaluation. The four groups were compared by a two-way analysis of variance. By using this approach, each analysis of variance yielded three p values (Table 3). The p value for the comparison labeled N/H refers to differences between the entire normocholesterolemic and the entire hypercholesterolemic group, whereas the comparison −/+ compares all nonatherosclerotic with all atherosclerotic patients. The third p value determines whether hypercholesterolemia and atherosclerosis have a significant interactive effect on each dependent variable (L/A). Two-sample comparisons between values obtained before and after EPA administration were made with a paired-sample t-test.

RESULTS

Comparison of platelet lipid composition in normal LDL-cholesterolemic and high LDL-cholesterolemic subjects. The PC/Chol ratio of platelets was significantly ($F(1,40) = 4.93$, $p < 0.05$) lower in high LDL-cholesterolemic (≥170 mg/dl) subjects than that in normal LDL-cholesterolemic subjects (Table 3). The phosphatidylinositol (PI)/Chol ratio also was significantly ($F(1,40) = 5.21$, $P < 0.05$) lower in high LDL-cholesterolemic subjects than that in normal LDL-cholesterolemic subjects. The other parameters of platelet lipid in high LDL-cholesterolemic subjects were not significantly different than those in normal LDL-cholesterolemic subjects.

Comparison of platelet lipid composition in subjects with and without atherosclerotic disease. Platelets from

TABLE 3

Lipid Composition of Platelets in Subjects with and without Atherosclerotic Disease, with and without High Plasma Low Density Lipoprotein (LDL)-Cholesterol (≥170 mg/dl)

	LDL-Cholesterol <170 mg/dl		LDL-Cholesterol ≥170 mg/dl				
	Atherosclerotic disease		Atherosclerotic disease		Significance[b]		
Lipid composition[a,c]	− (n = 11)	+ (n = 11)	− (n = 11)	+ (n = 11)	N/H	−/+	L/A
Chol/protein (×10) (w/w)	0.511 ± 0.122	0.523 ± 0.104	0.500 ± 0.065	0.546 ± 0.121	NS	NS	NS
PL/protein (w/w)	0.176 ± 0.045	0.159 ± 0.020	0.159 ± 0.031	0.153 ± 0.044	NS	NS	NS
LPC/protein (w/w)	0.002 ± 0.001	0.005 ± 0.007	0.003 ± 0.006	0.003 ± 0.005	NS	NS	NS
PC/protein (w/w)	0.068 ± 0.015	0.055 ± 0.011	0.056 ± 0.008	0.054 ± 0.020	NS	NS	NS
SM/protein (w/w)	0.032 ± 0.011	0.030 ± 0.005	0.031 ± 0.010	0.030 ± 0.007	NS	NS	NS
PI/protein (w/w)	0.008 ± 0.004	0.007 ± 0.003	0.006 ± 0.002	0.006 ± 0.004	NS	NS	NS
PS/protein (w/w)	0.016 ± 0.006	0.014 ± 0.003	0.015 ± 0.003	0.014 ± 0.005	NS	NS	NS
PE/protein (w/w)	0.046 ± 0.011	0.042 ± 0.006	0.040 ± 0.005	0.039 ± 0.013	NS	NS	NS
PL/cholesterol (mol/mol)	1.727 ± 0.225	1.564 ± 0.367	1.591 ± 0.285	1.413 ± 0.295	NS	NS	NS
LPC/cholesterol (mol/mol)	0.016 ± 0.013	0.050 ± 0.056	0.029 ± 0.047	0.037 ± 0.059	NS	NS	NS
PC/cholesterol (mol/mol)	0.675 ± 0.121	0.550 ± 0.148	0.564 ± 0.107	0.488 ± 0.136	<0.05	<0.025	NS
SM/cholesterol (mol/mol)	0.308 ± 0.062	0.295 ± 0.059	0.316 ± 0.093	0.285 ± 0.088	NS	NS	NS
PI/cholesterol (mol/mol)	0.075 ± 0.018	0.073 ± 0.026	0.063 ± 0.018	0.053 ± 0.029	<0.05	NS	NS
PS/cholesterol (mol/mol)	0.154 ± 0.032	0.137 ± 0.036	0.151 ± 0.035	0.127 ± 0.031	NS	NS	NS
PE/cholesterol (mol/mol)	0.454 ± 0.099	0.412 ± 0.092	0.406 ± 0.067	0.357 ± 0.078	NS	NS	NS
PC/SM (w/w)	2.24 ± 0.42	1.83 ± 0.39	1.86 ± 0.39	1.80 ± 0.49	NS	NS	NS

[a] Values are presented as mean ± SD.

[b] N/N = significance between all normal LDL-cholesterolemic (<170 mg/dl) and high LDL-cholesterolemic (≥170 mg/dl) subjects (independent of classification according to atherosclerotic disease); −/+ = significance between all subjects without atherosclerotic disease and those with atherosclerotic disease (independent of classification according to the levels of plasma LDL-Cholesterol); L/A = interaction between the effects of plasma LDL-Cholesterol level and atherosclerotic disease; NS = not significant.

[c] Abbreviations: Chol, cholesterol; PL, phospholipid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine.

subjects with atherosclerotic disease had a significantly ($F(1,40) = 6.70$, $p < 0.025$) lower PC/Chol ratio than those from subjects without atherosclerotic disease. The other parameters of platelets in subjects with atherosclerotic disease were not significantly different than those in subjects without atherosclerotic disease. The PC/protein (prot) ratio ($F(1,40) = 3.29$), the PL/Chol ratio ($F(1,40) = 3.94$), the phosphatidylserine (PS)/Chol ratio ($F(1,40) = 4.06$), the phosphatidylethanolamine (PE)/Chol ratio ($F(1,40) = 3.21$), and the PC/sphingomyelin (SM) ratio ($F(1,40) = 3.28$) tended to decrease in platelets from subjects with atherosclerotic disease ($0.05 < p < 0.10$) (Table 3).

Effect of EPA administration on plasma lipids in patients with atherosclerotic disease. Changes of plasma lipids after EPA administration are shown in Figure 1. Plasma levels of total Chol and LDL-Chol decreased ($p < 0.01$ and $p < 0.05$, respectively) after EPA administration. Plasma levels of TG and HDL-Chol did not change with EPA administration.

Effect of EPA administration on platelet lipid composition in patients with atherosclerotic disease. The PC/Chol ratio of platelets increased significantly ($p < 0.02$) from 0.483 ± 0.133 to 0.608 ± 0.127 (mean $\pm$ SD) after EPA administration (Table 4, Figure 2). The PC/prot ratio and the PL/Chol ratio also increased ($p < 0.05$) with EPA ingestion. The other characteristics did not change significantly. The PL/prot ratio, the SM/Chol ratio and the PE/Chol ratio tended to increase with EPA administration ($0.05 < p < 0.10$).

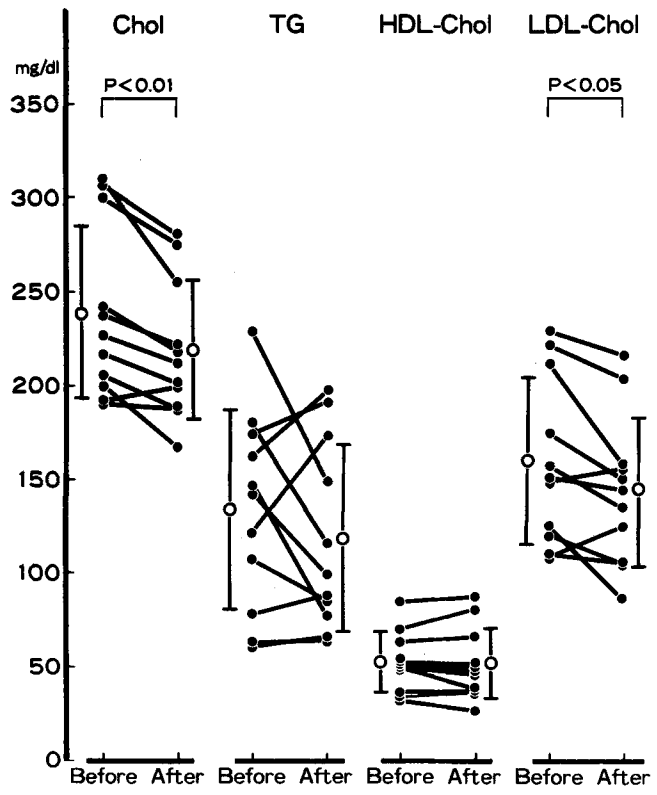

FIG. 1. Plasma lipid levels in patients with atherosclerotic disease before and after eicosapentaenoic acid ethyl ester (EPA-E) administration (Chol, cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein).

TABLE 4

Lipid Composition of Platelets in Patients with Atherosclerotic Disease before and after Purified Eicosapentaenoic Acid Ethyl Ester (EPA-E) Administration

Lipid composition [a,b]	Before	After
Chol/protein (×10) (w/w)	0.551 ± 0.107	0.513 ± 0.112
PL/protein (w/w)	0.148 ± 0.027	0.167 ± 0.033
LPC/protein (w/w)	0.004 ± 0.006	0.004 ± 0.005
PC/protein (w/w)	0.052 ± 0.013	0.061 ± 0.013[c]
SM/protein (w/w)	0.028 ± 0.005	0.031 ± 0.007
PI/protein (w/w)	0.007 ± 0.003	0.007 ± 0.003
PS/protein (w/w)	0.014 ± 0.003	0.014 ± 0.004
PE/protein (w/w)	0.040 ± 0.009	0.044 ± 0.008
PL/cholesterol (mol/mol)	1.369 ± 0.268	1.640 ± 0.235[c]
LPC/cholesterol (mol/mol)	0.034 ± 0.042	0.033 ± 0.044
PC/cholesterol (mol/mol)	0.483 ± 0.133	0.608 ± 0.127[d]
SM/cholesterol (mol/mol)	0.254 ± 0.039	0.302 ± 0.057
PI/cholesterol (mol/mol)	0.063 ± 0.024	0.069 ± 0.022
PS/cholesterol (mol/mol)	0.127 ± 0.034	0.140 ± 0.032
PE/cholesterol (mol/mol)	0.372 ± 0.091	0.430 ± 0.062
PC/SM (w/w)	1.89 ± 0.37	2.04 ± 0.40

[a] Values are presented as mean ± SD.

[b] Abbreviations: Chol, cholesterol; PL, phospholipid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine.

[c] Significantly different from before EPA-E administration at $p < 0.05$ (Student's t-test; paired).

[d] Significantly different from before EPA-E administration at $p < 0.02$ (Student's t-test; paired).

Effect of EPA administration on platelet fatty acid composition in patients with atherosclerotic disease. Relative content of each fatty acid is expressed as wt% of total fatty acids in total lipids (Table 5). The content of EPA (C20:5) in platelet lipid increased in all patients, and mean EPA content increased ($p < 0.001$) from $2.3 \pm 1.1\%$ (mean $\pm$SD) to $3.9 \pm 1.1\%$ with EPA administration. Arachidonic acid (AA) (C20:4) content decreased ($p < 0.01$). Consequently, the ratio of EPA to AA (20:5/20:4) in platelet lipid increased ($p < 0.001$) from 0.098 ± 0.047 to 0.185 ± 0.057 after ingestion of EPA ethyl ester. DPA (C22:5) content also increased significantly ($p < 0.01$); however, docosahexaenoic acid (DHA) (C22:6) content was not changed by EPA administration.

DISCUSSION

In this study, molar ratio of PL to Chol was not different in platelets from hypercholesterolemic and normocholesterolemic subjects, in agreement with the report by Jakubowski et al. (25). The PC/Chol ratio in platelets from hypercholesterolemic subjects was significantly decreased, compared with normocholesterolemic subjects.

Platelets from subjects with atherosclerotic disease had changes in lipid composition similar to those from hypercholesterolemic subjects. The PL/Chol ratio was not decreased in platelets from subjects with atherosclerotic disease, which is similar to the findings of Shattil et al. (26). This study extends their observations; we demonstrated a significant decrease of the PC/Chol ratio in platelets from subjects with atherosclerotic disease.

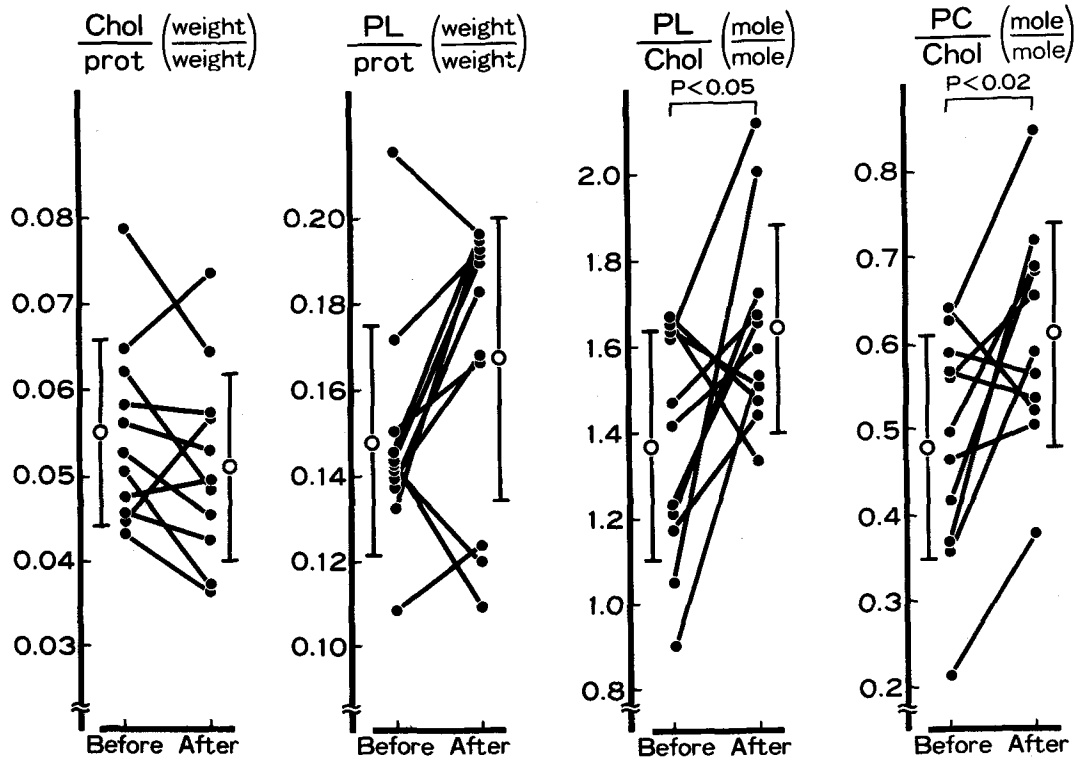

FIG. 2. Lipid composition of platelets in patients with atherosclerotic disease before and after eicosapentaenoic acid ethyl ester (EPA-E) administration (PL, phospholipids; Chol, cholesterol; prot, protein; PC, phosphatidylcholine).

TABLE 5

Fatty Acid Composition of Platelets in Patients with Atherosclerotic Disease before and after Purified Eicosapentaenoic Acid Ethyl Ester (EPA-E) Administration

Fatty acid[a]	Before	After
16:0	19.5 ± 3.1	19.4 ± 2.1
18:0	17.0 ± 1.6	17.7 ± 1.7
18:1 (ω-9)	17.3 ± 1.3	17.7 ± 0.6
18:2 (ω-6)	7.0 ± 1.6	6.5 ± 1.4
20:4 (ω-6)	23.9 ± 3.5	21.1 ± 2.4[b]
20:5 (ω-3)	2.3 ± 1.1	3.9 ± 1.1[c]
22:5 (ω-3)	2.3 ± 1.2	3.1 ± 1.4[b]
22:6 (ω-3)	4.3 ± 1.3	4.3 ± 2.0
Others	6.5 ± 2.4	6.4 ± 1.9
20:5/20:4 (×100)	9.8 ± 4.7	18.5 ± 5.7[c]

[a]Fatty acids are denoted by ratio of chain length to number of double bonds. Values are expressed as percentage of total peak area and presented as mean ± SD.

[b]Significantly different from before EPA-E administration at $P < 0.01$ (Student's t-test; paired).

[c]Significantly different from before EPA-E administration at $P < 0.001$ (Student's t-test; paired).

The PC/free Chol ratio in plasma from patients with atheroselerotic disease also was found to be decreased. Kuksis et al. (27,28) proposed the ratio as an indicator for atherosclerosis. Several reports have shown similar changes in the composition of lipids from the atherosclerotic aorta. Böttcher et al. (29,30) investigated composition of lipids from aorta at different stages of atherosclerosis. Their data showed that the percentage of PL decreased, while free Chol increased, as atherosclerosis became more severe (29). In addition, they reported that the main feature of PL class composition of the atherosclerotic aorta is the increase in SM percentage in parallel with the degree of atherosclerosis, with corresponding decrease in PC content (30). These findings are in agreement with the changes in the lipid composition of platelets from patients with atherosclerotic disease, as shown in this study.

The parallel changes in the lipid composition of plasma, platelet and the arterial tissues do not necessarily establish a causal relationship, although one might assume that the changes in plasma precede those in the tissues (31). The accumulation of lipid within the vessel wall, that results in the development of the initial fatty atheromatous lesion, is usually preceded by hypercholesterolemia (32), and we observed changes in the platelet lipid composition of hypercholesterolemic patients similar to those of atherosclerotic patients.

The ratio of serum glycerophospholipids/free Chol, in particular, the PC/free Chol ratio, has been suggested as a possible indicator of asymmetric fluidity of lipoproteins in disease (33). Abnormalities of cell-membrane fluidity has been thought to be important in the pathogenesis of several diseases, because membrane fluidity affects cell behavior (5). Thus, the decreased PC/Chol ratio of platelets might be a physicochemical basis for the role of them in the pathogenesis of atherosclerosis.

The mechanism of the change of PC/Chol ratio in platelets from patients with atherosclerotic disease has

not been elucidated. This change in lipid composition of platelets might be partly secondary to the change in plasma from patients with ischemic vascular disease (27,34). Platelets cannot synthesize Chol (35), but they are capable of synthesizing and degrading PL (36,37). The change of PC/Chol ratio in platelets, therefore, might reflect the changes in PL metabolism in platelets themselves. In this study, we observed that the PL/Chol ratio and the PC content had a tendency to decrease in platelets from patients with atherosclerotic disease. The results indicate that enhanced degradation of PL, especially PC, might occur in platelets from patients with atherosclerotic disease. Indeed, Chol-rich platelets have an augmented capability of Thromboxane A_2 production (3), and phospholipase A_2 acting on PC is markedly enhanced in platelets with elevated Chol/PL ratio (38). Arachidonate also can be liberated by phospholipase C indirectly from PI via mediation of diglyceride lipase (39). In this context, it is interesting that the PI/Chol ratio decreased only in platelets from hypercholesterolemic patients.

The ratio of PC to Chol in platelet lipids from patients with atherosclerotic disease significantly increased with administration of 1.8 g/day of EPA ethylester for 4–10 wk. The duration of EPA administration (4–10 weeks) did not have a significant effect on the change in PC/Chol ratio. The mechanism of the increase in PC/Chol ratio by EPA administration has not been elucidated yet. Shepherd et al. (40) reported that the relative percentage Chol in the LDL fraction fell, while that of PL rose, during polyunsaturated fat feeding. Therefore, the increases in PL/Chol and PC/Chol ratio in platelets may be due, in part, to those in LDL. There are some reports that polyunsaturated fatty acids enhance PC synthesis (41, 42), and the PC/prot ratio increased with ingestion of EPA. Thus EPA must directly affect PL metabolism in platelets.

EPA content in platelet lipid in patients with atherosclerotic disease increased with administration of 1.8 g/day of EPA ethyl ester for 4–10 wk, while AA content decreased. These results, except for the AA decrease, are similar to the findings in healthy volunteers reported by Terano et al. (13). Goodnight et al. (43) proposed two mechanisms of reduction of AA during a fish diet. One mechanism is that the decreased dietary linoleic acid (most fish oils contain only 1% to 2% linoleic acid) may lead to reduced arachidonate levels in plasma and, hence, platelet membrane PL. The other is that AA simply may be displaced by other fatty acid. In our study, the patients did not change their dietary habits and the linoleic acid (C18:2) content in platelet lipid did not change significantly; therefore, AA was probably replaced by EPA and DPA. The DHA content of platelets in patients with atherosclerotic disease was unchanged, while the DPA content increased after EPA administration. Therefore, the ability exists for elongation of EPA into DPA, but DPA cannot be desaturated into DHA.

In conclusion, this report establishes that EPA increases the PL/Chol ratio and the PC/Chol ratio of platelets from patients with atherosclerosis. These ratio shifts, in addition to fatty acyl chain alterations, might increase the membrane fluidity of platelets from patients with atherosclerosis. This observation may demonstrate another benefit of EPA administration to patients with atherosclerotic disease.

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REFERENCES

1. Weksler, B.B., and Nachman, R.L. (1981) *Am. J. Med. 71,* 331–333.
2. Kuo, P.T. (1981) *Am. Heart J. 102,* 949–953.
3. Stuart, M.J., Gerrard, J.M., and White, J.G. (1980) *N. Engl. J. Med. 302,* 6–10.
4. Shattil, S.J., and Cooper, R.A. (1976) *Biochemistry 15,* 4832–4837.
5. Cooper, R.A. (1977) *N. Engl. J. Med. 297,* 371–377.
6. Carvalho, A.C.A., Colman, R.W., and Lees, R.S. (1974) *N. Engl. J. Med. 290,* 434–438.
7. Mustard, J.F., and Packhem, M.A. (1969) *Circulation 40, Suppl. 4,* 20–30.
8. Knapp, H.R., Reilly, I.A.G., Alessandrini, P., and FitzGerald, G.A. (1986) *N. Engl. J. Med. 314,* 937–942.
9. Dyerberg, J., Bang, H.O., Stoffersen, E., Moncada, S., and Vane, J.R. (1978) *Lancet ii,* 117–119.
10. Dyerberg, J., and Bang, H.O. (1979) *Lancet ii,* 433–435.
11. Siess, W., Roth, P., Scherer, B., Kurzmann, I., Böhlig, B., and Weber, P.C. (1980) *Lancet i,* 441–444.
12. Sanders, T.A.B., Vickers, M., and Haines, A.P. (1981) *Clin. Sci. 61,* 317–324.
13. Terano, T., Hirai, A., Hamazaki, T., Kobayashi, S., Fujita, T., Tamura, Y., and Kumagai, A. (1983) *Atherosclerosis 46,* 321–331.
14. Nagakawa, Y., Orimo, H., Harasawa, M., Morita, I., Yashiro, K., and Murota, S. (1983) *Atherosclerosis 47,* 71–75.
15. Friedewald, W.T., Levy, R.I., and Fredrickson, D.S. (1972) *Clin. Chem. 18,* 499–502.
16. Hoeg, J.M., Gregg, R.E., and Brewer, Jr. H.B. (1986) *J. Am. Med. Assoc. 255,* 512–521.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
18. Folch, J., Less, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497–509.
19. Rudel, L.L., and Morris, M.D. (1973) *J. Lipid Res. 14,* 364–366.
20. Chen, P.S. Jr., Toribara, T.Y., and Waner, H. (1956) *Anal. Chem. 28,* 1756–1758.
21. Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W., and Fu, P.C. (1974) *Clin. Chem. 20,* 470–475.
22. Fletcher, M.J. (1968) *Clin. Chim. Acta 22,* 393–397.
23. Srinivasan, S.R., Ellefson, R.D., Whitaker, C.F., and Berenson, G.S. (1978) *Clin. Chem. 24,* 157–161.
24. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) *Lipids 5,* 494–496.
25. Jakubowski, J.A., Ardlie, N.G., and Nestel, P.J. (1980) *Prostaglandins Med. 5,* 457–467.
26. Shattil, S.J., Bennett, J.S., Colman, R.W., and Cooper, R.A. (1977) *J. Lab. Clin. Med. 89,* 341–353.
27. Kuksis, A., Myher, J.J., Geher, K., Jones, G.J.L., Breckenridge, W.C., Feather, T., Hewitt, D., and Little, J.A. (1982) *Arteriosclerosis 2,* 296–302.
28. Kuksis, A., Roberts, A., Thompson, J.S., Myher, J.J., and Geher, K. (1983) *Arteriosclerosis 3,* 389–397.
29. Böttcher, C.J.F., Woodford, F.P., Ter Haar Romeny, C.C.H., Boelsma, E., and Van Gent, C.M. (1959) *Nature 183,* 47–48.
30. Böttcher, C.J.F., and Van Gent, C.M. (1961) *J. Atheroscler. Res. 1,* 36–46.
31. Portman, O.W., and Alexander, N. (1972) *Biochim. Biophys. Acta 260,* 460–474.
32. Connor, W.E. (1979) in *The Biochemistry of Atherosclerosis* (Scanu, A.M., Wissler, R.W., and Getz, G.S., eds.) pp. 371–418, Marcel Dekker, New York, NY.
33. Casu, A. (1979) *Ital. J. Biochem. 28,* 26–38.
34. Shattil, S.J., Anaya-Galindo, R., Bennett, J., Colman, R.W., and Cooper, R.A. (1975) *J. Clin. Invest. 55,* 636–643.
35. Derksen, A., and Cohen, P. (1973) *J. Biol. Chem. 248,* 7396–7403.
36. Goracci, G., Gresele, P., Arienti, G., Porrovecchio, P., Nenci, G.G., and Porcellati, G. (1983) *Lipids 18,* 179–185.

37. Billah, M., Lapetina, E.G., and Cuatrecasas, P. (1980) *J. Biol. Chem. 255*, 10227–10231.
38. Kramer, R.M., Jakubowski, J.A., Vaillancourt, R., and Deykin, D. (1982) *J. Biol. Chem. 257*, 6844–6849.
39. Rittenhouse-Simmons, S. (1979) *J. Clin. Invest. 63*, 580–587.
40. Shepherd, J., Packard, C.J., Grundy, S.M., Yeshurun, D., Gotto, A.M., Jr., and Taunton, O.D. (1980) *J. Lipid Res. 21*, 91–99.
41. Pelech, S.K., and Vance, D.E. (1984) *Biochim. Biophys. Acta 779*, 217–251.
42. Pelech, S.L., Cook, H.W., Paddon, H.B., and Vance, D.E. (1984) *Biochim. Biophys. Acta 795*, 433–440.
43. Goodnight, S.H. Jr., Harris, W.S., Connor, W.E., and Illingworth, D.R. (1982) *Arteriosclerosis 2*, 87–113.

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Boreal Freshwater Fish Diet Modifies the Plasma Lipids and Prostanoids and Membrane Fatty Acids in Man

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The effect of fish diet on 43 healthy male students was studied. They ate a fish-containing meal for 15 weeks on an average of 3.7 times per week. Twenty-one of them voluntarily restricted their lipid intake while the rest ate normally. Controls continued their usual eating habits (19 students). The meals consisted of Finnish freshwater fish (87%) (vendace, pike, perch and rainbow trout) and brackish water fish (13%) (Baltic herring) that provided about 1 g of ω-3 polyunsaturated fatty acids per day (0.25 g eicosapentaenoic acid and 0.55 g docosahexaenoic acid). During the diet, ω-3 fatty acids increased in erythrocyte ghosts and platelets at the expense of ω-6 fatty acids. The concentration of serum cholesterol diminished in those fish consumers who lowered their lipid intake. Apolipoprotein A_1 and B were lowered in both fish-consuming groups. Triglyceride levels also showed a tendency to decrease. The formation of thromboxane B_2 during incubation of whole blood decreased in both fish-consuming groups. The decrease of plasma 6-keto-$PGF_{1\alpha}$ was not statistically significant, if compared with the controls. The results obtained indicate that a moderate intake of fish-containing meals has some beneficial effects on the plasma lipid and prostanoid metabolism, when coronary heart disease risk factors are considered.
Lipids 23, 924–929 (1988).

There are several reports on the consumption of fish or their oils and their positive effects on coronary disease risk factors. Eating fish lowers serum triglycerides (1) and cholesterol (2). Furthermore, the aggregation of platelets diminishes (3) and thromboxane production is inhibited (4). The bleeding time is prolonged (5). Blood viscosity is lowered (6) and the deformability of red cells is increased (7). Many of these positive effects, however, appear to require rather high intakes of fish oil. There are three epidemiological reports of positive effects, on coronary heart disease risk factors, by a modest fish-consuming diet with an affluent western type of diet (8–10). Furthermore, two reports have been published with negative results (11,12). Consumption of freshwater fish has been studied much less, and no reports of controlled studies of the effects on coronary heart disease risk factors are available. We have found that, at least in the boreal region of Europe, quite significant amounts of ω-3 fatty acids occur in freshwater fish tissues (13). This suggests that, by eating freshwater fish meals, it also might be possible to obtain positive effects against risk factors. The aim of this study was to determine if such effects could be seen in male students who were offered one fish meal during working days over a period of ca. 100 days. The meals were planned so that they could be prepared in public restaurants using readily available materials within the price range for normal operation. The results indicate that such meals can cause some positive changes in lipid metabolism in an area like Eastern Finland where high coronary morbidity and mortality are prevalent (14).

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Abbreviations: EDTA, ethylenediamine tetraacetic acid; 6-keto-$PGF_{1\alpha}$, 6-ketoprostaglandin $F_{1\alpha}$.

SUBJECTS AND MEALS

Healthy male students (n = 62) voluntarily participated in the study. The study was approved by the Ethical Committee of the University of Kuopio. None of the students exhibited allergies to fish. The participants' anthropometric properties are in Table 1.

Following base-line measurements, the students were randomly divided into three groups by drawing letters generated by a random-number generator. There were 19 students in the control group; the remainder were provided with a fish meal every working day, and 21 were furthermore asked to voluntarily diminish their fat intake, especially saturated-fat dairy products. All students in the study received a meal (free of charge) once per working day for 103 days. Food consumption was recorded.

The fish were prepared by usual cooking methods used in public catering systems including frying and baking. The same recipe was used only twice during the study. The portion of fish served per meal was 180 g. The average amount of ω-3 fatty acids was estimated to be about 1.8 g per meal (range = 0.6–3.0 g). One fish meal per two weeks was served to the control group, according to the normal frequency of fish meals in the university restaurant.

TABLE 1

The Anthropometric Data and Starting Blood Pressures of Male Students Participating in the Study (means ± SD)

	Controls	Fish eaters	Fish eaters with reduced fat intake
Number of students	19	22	21
Age	23 ± 2	22 ± 3	22 ± 2
Length (cm)	181 ± 5	178 ± 5	181 ± 5
Weight (kg)	74 ± 8	71 ± 7	71 ± 6
Body mass index (kg/m^2)	22.6 ± 1.8	22.3 ± 1.9	21.6 ± 1.8
Systolic blood pressure (mm Hg)	130 ± 15	125 ± 13	126 ± 16
Diastolic blood pressure (mm Hg)	83 ± 10	78 ± 9	83 ± 12

The compliance of the students to a dietary regimen was followed. The fish-consuming groups showed somewhat higher compliance (76%) than the control group (68%). On an average, the fish-eating groups consumed 3.7 ± 0.8 fish meals per week and about 1 g of ω-3 fatty acids per day, which included 0.25 g eicosapentaenoic and 0.55 g docosahexaenoic acid. The meals were found to be palatable. The only reasons for dropping out of the study were inability to remain in the city and other similar reasons. The controls ate, on an average, 0.25 fish-containing meals per week.

The blood samples were drawn before the fish diet was started and, again, when it had been eaten for 7 and 15 weeks. These three samples were taken and analyzed satisfactorily from 13–15 subjects of each group. Furthermore, additional samples were collected in the following autumn when the students had been consuming a usual diet for about five months. These samples could not be taken earlier because of the summer holidays.

METHODS

Before the blood samples were taken, the students were advised not to consume alcohol or acetylsalicylic acid for seven days and to avoid heavy exercise for three days. During the morning before the collection of blood samples, the students did not eat breakfast. The blood samples were drawn before, during (7 and 15 wk) and after (20 wk) the study, between 7:30 and 10:30 AM, after each student had been lying on a bed, totally relaxed for 30 min. All of the blood samples were placed in vacuum tubes without a tourniquet, by the same nurse in the following order: the first two 5-ml samples for serum (apolipoprotein, cholesterol, triglyceride and selenium analyses), the next two 5-ml samples were drawn into Terumo Venoject tubes containing dipotassium-EDTA (5 mg) and acetylsalicylic acid (0.09 mg) (6-keto-$PGF_{1\alpha}$ determinations), two 5-ml samples into EDTA-tubes (preparation of platelets and erythrocyte ghosts for fatty acid analyses), one 5-ml sample into a precooled Terumo Venoject tube (thromboxane B_2 analysis) and one 5-ml sample into a EDTA-tube for the analysis of hemoglobin and blood picture (measured with the aid of a Coulter Counter). The most important purpose of this particular sampling order, and the limitations before drawing samples, was to minimize errors in the very sensitive prostanoid determinations.

The samples for thromboxane B_2 determination were transferred immediately into a water bath (37°C) for 30 min, after which the tubes were placed in ice bath and spun at 4°C for 15 min at 2,000 *g* to separate the serum. The 6-keto-$PGF_{1\alpha}$ samples were spun in the same way to get the plasma.

All the plasma and serum samples were stored at −80°C until analyzed.

The platelets and erythrocyte ghosts were separated for fatty acid analysis. Platelet-rich plasma was obtained by spinning the blood samples at low speed (120 *g* for 10 min at 4°C). The platelets were separated by centrifugation at 2,000 *g* for 15 min (4°C) and, then washed with TRIS-HCl buffer (pH 7.6, 172 mmol/l). Erythrocytes were washed with the same buffer and hemolyzed in the same diluted buffer (11 mmol/l). Ghosts were prepared by centrifugation at 20,000 *g* for 20 min at 4°C. Both platelet and erythrocyte ghost sediments were sonicated in the TRIS-HCl buffer (172 mmol/l) and stored at −80°C until analyzed.

The serum cholesterol and triglyceride concentrations were determined with a Hitachi 705 Analyzer using enzymatic methods (Boehringer Mannheim, GmbH, Mannheim, FRG). Apolipoprotein A_1 and B were measured by immunoturbidometry using antisera obtained from Orion Diagnostica (Orion Ltd, Espoo, Finland). Standardization of the apolipoprotein determination was performed as described earlier (15).

The fatty acids of the platelets and erythrocyte ghosts were transmethylated with sodium methoxide and analyzed by capillary gas-liquid chromatography (Carlo Erba Fractovap 2350, Milano, Italy) in a 15 m OV-351 fused-silica capillary column (Nordion Instruments Ltd, Helsinki, Finland) as described earlier (13). Samples taken 20 weeks after the programmed meals were pooled within the groups and no fatty acid analyses were made from the individual samples.

The 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 were isolated and purified using Sep-pak C 18 cartridges (Waters Associates, USA) (16). Their concentrations were measured by radioimmunoassay kits (New England Nuclear, USA). These use iodinated analogs of these prostanoids as tracers. The corresponding metabolites (tyrosyl methyl esters) labeled with ^{125}I were added to the samples to monitor the recovery.

The rubidium efflux through the erythrocyte cell membrane was measured as described earlier (17).

The data obtained were statistically analyzed by using nonparametric methods. Wilcoxon matched-pairs, signed-ranks test was used for the comparisons inside the groups and Mann-Whitney U-test between the groups.

RESULTS

The serum triglyceride and apolipoprotein A_1 and B levels decreased, during the study, in most students on the fish diet. However, changes in the means were small, due to a couple of very exceptional values. Thus, the significance at $p < 0.1$ was used in Table 2 to indicate the tendencies. The serum cholesterol level decreased in those fish eaters who voluntarily reduced their fat intake. Those fish eaters who continued their usual eating habits, had similar cholesterol levels as the controls at the end of the fish diet. The high basal level of cholesterol in the control group is caused mostly by two subjects with exceptionally high values (>8 mmol/l), which decreased during the study. The changes in the serum cholesterol and triglyceride values during the diet correlated strongly with the changes of the apolipoprotein B. Five months after the fish-eating period, no differences were observed between the groups. The only exception was the apolipoprotein A_1, which still remained at lowered level in the fish-diet group with no limitations of fat intake.

The shifts in fatty acids in the platelets and erythrocyte ghosts, due to fish-containing meals, are summarized in Tables 3 and 4. No significant changes were observed in the controls. The content of arachidonic (20:4ω6) and docosatetranoic (22:4ω6) acids decreased with the increase of eicosapentaenoic (20:5ω3) and docosahexaenoic (22:6ω3) acid contents in both particles. Furthermore, a decrease was observed in the content of dihomogamma-

TABLE 2

Serum Triglyceride, Cholesterol and Apolipoprotein A_1 and B Levels in Male Students Consuming an Average of 3.7 Fish Meals/Week up to 15 Weeks (means ± SD, n = number of students)

	Predietary period	Dietary period	
		7 weeks	15 weeks
Controls, n = 12			
Triglycerides (mmol/l)	0.86 ± 0.20	0.98 ± 0.23	0.95 ± 0.30
Cholesterol (mmol/l)	5.14 ± 1.12	5.03 ± 0.81	4.82 ± 0.73
Apolipoprotein A_1 (g/l)	1.27 ± 0.17	1.27 ± 0.15	1.22 ± 0.13
Apolipoprotein B (g/l)	0.69 ± 0.07	0.68 ± 0.13	0.67 ± 0.10
Fish eaters, n = 14			
Triglycerides (mmol/l)	0.97 ± 0.31	1.02 ± 0.44	0.89 ± 0.30[a,d]
Cholesterol (mmol/l)	4.49 ± 0.56	4.86 ± 0.69[a]	4.86 ± 0.76[a]
Apolipoprotein A_1 (g/l)	1.26 ± 0.16	1.16 ± 0.17[b]	1.07 ± 0.15[c,e]
Apolipoprotein B (g/l)	0.70 ± 0.12	0.69 ± 0.09	0.66 ± 0.12[a]
Fish eaters with voluntary restriction of fat intake, n = 15			
Triglycerides (mmol/l)	0.86 ± 0.26	0.81 ± 0.27	0.78 ± 0.26[a,d]
Cholesterol (mmol/l)	4.49 ± 0.62	4.46 ± 0.64	4.25 ± 0.82[f]
Apolipoprotein A_1 (g/l)	1.23 ± 0.15	1.20 ± 0.16	1.15 ± 0.16[f]
Apolipoprotein B (g/l)	0.63 ± 0.10	0.62 ± 0.10	0.58 ± 0.10[f]

[a] Significant at $p < 0.1$,

[b] Significant at $p < 0.01$,

[c] Significant at $p < 0.001$, when compared with initial level (Wilcoxon matched-pairs, signed-ranks test).

[d] Significant at $p < 0.05$,

[e] Significant at $p < 0.001$, when the change in 15 weeks is compared with controls (Mann-Whitney U-test).

[f] Significant at $p < 0.05$, when compared with initial level (Wilcoxon matched-pairs, signed-ranks test).

linolenic acid (20:3ω6) and oleic acid (18:1ω9) in erythrocyte ghosts. Only minor changes were observed in the other fatty acids. The changes in the polyunsaturated fatty acids took place faster in the platelets than in the erythrocyte ghosts (Fig. 1). When the students had been on their usual diets for 5 months after the fish regimen, the proportions of the fatty acids had returned to prestudy levels.

The thromboxane B_2 production during the incubation of the blood samples decreased significantly during the 15 weeks on the fish diet (Table 5). During the first seven weeks, the decrease did not reach the level of statistical significance. The decrease was similar in lipid restricted and nonrestricted fish diet. In the control group, a slight decrease also was observed during the follow-up period, but this was not statistically significant.

During the fish diet, the plasma level of 6-keto-$PGF_{1\alpha}$ showed a decrease that was statistically significant after 15 weeks (Table 5). A slight decrease also was seen in the control group.

The flux of rubidium through the erythrocyte cell membrane was measured at the end of the fish regimen. The rate constant of rubidium efflux was slightly greater in the fish-eating group ($5.99 + 0.61 \times 10^3$ min^{-1}, n = 9) than in the controls ($5.66 + 0.84 \times 10^3$ min^{-1}, n = 8), but the difference did not reach the level of statistical significance.

No changes in the blood picture were observed.

DISCUSSION

The results of this study indicate that freshwater fish contain adequate amounts of ω-3 polyunsaturated fatty acids to permit demonstration of distinct effects in the platelet and erythrocyte ghosts, as well as in the metabolism of these acids, when consumed over a 15-week period. The freshwater fish tissues contain relatively large amounts of ω-3 polyunsaturated fatty acids (13,18), as do marine fish (19). There are several reports on the effects of the marine fish diets on the fatty acid patterns and their metabolites in human populations (20). Thus, both kinds of fish are effectively utilized in the human body.

The proportions of eicosapentaenoic and docosahexaenoic acids increased both in the erythrocyte ghosts and platelets, as was expected. Changes in the erythrocyte ghosts in 15 weeks were similar, as reported earlier by Sanders et al. (21), with essentially higher intake of ω-3 fatty acids for six weeks (20 ml cod-liver oil per day containing 1.8 g eicosapentaenoic and 2.2 g docosahexaenoic

TABLE 3

The Effect of Fish Diet on the Platelet Fatty Acid Composition of Male Students in 15 Weeks (mol% of total, means ± SD)

Fatty acids	Predietary period	Dietary period	
		7 weeks	15 weeks
Fish eaters, n = 14			
16:0	15.7 ± 1.4	15.7 ± 1.5	15.7 ± 1.5
18:0	19.0 ± 1.4	18.8 ± 1.5	19.0 ± 1.1
20:0	0.9 ± 0.3	1.0 ± 0.3	0.9 ± 0.3
Saturated	35.6 ± 1.1	35.5 ± 1.6	35.6 ± 1.6
16:1ω7	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.2
18:1ω9	15.6 ± 1.2	15.5 ± 0.9	15.6 ± 0.8
20:1ω9+11	1.0 ± 0.3	1.1 ± 0.3	1.1 ± 0.3
Monoenes	17.2 ± 1.3	17.2 ± 1.0	17.3 ± 1.0
18:2ω6	7.9 ± 1.8	8.0 ± 1.2	7.8 ± 1.1
18:3ω3	0.6 ± 0.3	0.8 ± 0.3[a]	0.7 ± 0.3
20:2ω6	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
20:3ω6	2.8 ± 0.5	3.1 ± 0.5	2.8 ± 0.7
20:4ω6	26.6 ± 2.9	24.7 ± 2.3[b]	24.5 ± 2.1[c]
20:5ω3	1.2 ± 0.6	1.9 ± 1.0[c]	2.4 ± 1.2[c]
22:4ω6	2.0 ± 0.3	1.8 ± 0.3[b]	1.6 ± 0.3[b]
22:5ω3	2.0 ± 0.4	2.1 ± 0.3	2.1 ± 0.3
22:6ω3	3.3 ± 0.8	4.1 ± 0.7[b]	4.5 ± 0.7[c]
Polyunsaturated	47.2 ± 1.7	47.3 ± 1.8	47.1 ± 2.1
ω-3	7.2 ± 1.3	8.9 ± 1.6[c]	9.7 ± 1.9[c]
ω-6	40.0 ± 2.2	38.4 ± 1.9[b]	37.4 ± 2.5[c]
ω-3/ω-6	0.18 ± 0.05	0.23 ± 0.05[c]	0.26 ± 0.07[c]
Fish eaters with voluntary restriction of fat intake, n = 15			
16:0	14.6 ± 0.8	15.1 ± 0.9[a]	15.1 ± 1.0
18:0	19.1 ± 0.7	19.1 ± 0.7	19.3 ± 1.0
20:0	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.2
Saturated	34.6 ± 0.9	35.1 ± 1.1	35.3 ± 1.3
16:1ω7	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
18:1ω9	15.3 ± 0.6	15.5 ± 0.8	15.4 ± 1.1
20:1ω9+11	0.8 ± 0.1	1.0 ± 0.2[b]	1.1 ± 0.2[b]
Monoenes	16.6 ± 0.7	16.9 ± 0.9	16.9 ± 1.2
18:2ω6	7.9 ± 1.3	8.0 ± 1.3	8.0 ± 1.1
18:3ω3	0.6 ± 0.2	0.7 ± 0.3	0.7 ± 0.2[a]
20:2ω6	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
20:3ω6	2.9 ± 0.6	2.8 ± 0.7	2.6 ± 0.5[a]
20:4ω6	28.0 ± 1.4	26.0 ± 2.0[c]	25.4 ± 1.6[c]
20:5ω3	1.1 ± 0.3	1.8 ± 0.4[c]	1.9 ± 0.4[c]
22:4ω6	2.2 ± 0.4	1.9 ± 0.4[c]	1.8 ± 0.3[c]
22:5[3]	2.1 ± 0.4	2.0 ± 0.3	2.1 ± 0.3
22:6ω3	3.3 ± 0.5	4.2 ± 0.4[c]	4.6 ± 0.6[c]
Polyunsaturated	48.8 ± 1.3	48.0 ± 1.6[b]	47.8 ± 2.2[a]
ω-3	7.1 ± 0.7	8.7 ± 0.8[c]	9.3 ± 1.0[c]
ω-6	41.7 ± 1.1	39.3 ± 1.4[c]	38.5 ± 2.1[c]
ω-3/ω-6	0.17 ± 0.02	0.22 ± 0.02[c]	0.24 ± 0.03[c]

[a]Significant at $p < 0.05$,
[b]Significant at $p < 0.01$,
[c]Significant at $p < 0.001$, when compared with initial level.

TABLE 4

The Effect of Fish Diet on the Fatty Acid Composition in Erythrocyte Ghosts of Male Students in 15 Weeks (mol% of total, means ± SD)

Fatty acids	Predietary period	Dietary period	
		7 weeks	15 weeks
Fish eaters, n = 14			
16:0	19.2 ± 1.9	19.3 ± 1.9	19.1 ± 1.1
18:0	16.5 ± 0.7	16.5 ± 0.7	16.3 ± 0.8
20:0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Saturated	35.9 ± 2.0	36.0 ± 2.0	35.6 ± 1.2
16:1ω7	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
18:1ω9	14.9 ± 0.7	14.9 ± 0.7	14.4 ± 0.7[a]
20:1ω9+11	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Monoenes	15.6 ± 0.8	15.7 ± 0.7	15.1 ± 0.7
18:2ω6	11.6 ± 1.6	11.2 ± 1.3	11.2 ± 1.4
18:3ω3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:2ω6	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:3ω6	2.4 ± 0.6	2.1 ± 0.5[a]	2.1 ± 0.6[a]
20:4ω6	19.1 ± 1.3	17.5 ± 1.6[b]	16.4 ± 1.4[c]
20:5ω3	1.6 ± 0.3	2.5 ± 0.8[c]	2.9 ± 1.0[c]
22:4ω6	2.7 ± 0.7	2.3 ± 0.7[a]	2.2 ± 0.6[a]
22:5ω3	3.2 ± 0.5	3.4 ± 0.7	3.5 ± 0.7
22:6ω3	7.2 ± 1.4	8.7 ± 1.3[c]	10.4 ± 1.2[c]
Polyunsaturated	48.5 ± 2.5	48.3 ± 2.6	49.3 ± 1.7
ω-3	12.3 ± 1.5	14.9 ± 2.6[c]	17.1 ± 2.5[c]
ω-6	36.2 ± 2.2	33.4 ± 2.5[c]	32.2 ± 2.3[c]
ω-3/ω-6	0.34 ± 0.05	0.45 ± 0.11[c]	0.54 ± 0.13[c]
Fish eaters with voluntary restriction of fat intake, n = 15			
16:0	18.2 ± 1.6	18.7 ± 2.0	18.6 ± 1.4
18:0	16.8 ± 0.6	16.8 ± 0.8	16.4 ± 0.8
20:0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Saturated	35.2 ± 1.4	35.7 ± 1.9	35.2 ± 1.2
16:1ω7	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
18:1ω9	15.3 ± 1.0	14.8 ± 0.8[a]	14.5 ± 0.7[c]
20:1ω9+11	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Monoenes	16.0 ± 1.0	15.5 ± 0.8[a]	15.3 ± 0.7[b]
18:2ω6	11.4 ± 1.0	11.3 ± 1.2	11.6 ± 1.2
18:3ω3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:2ω6	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:3ω6	2.5 ± 0.3	2.1 ± 0.4[b]	2.0 ± 0.4[c]
20:4ω6	19.0 ± 1.2	17.5 ± 1.2[c]	16.6 ± 1.4[c]
20:5ω3	1.6 ± 0.4	2.3 ± 0.4[c]	2.5 ± 0.5[c]
22:4ω6	2.7 ± 0.5	2.6 ± 0.6	2.4 ± 0.4[a]
22:5ω3	3.3 ± 0.6	3.2 ± 0.7	3.4 ± 0.4
22:6ω3	7.7 ± 1.8	9.2 ± 1.3[c]	10.4 ± 1.3[c]
Polyunsaturated	48.8 ± 2.1	48.8 ± 2.4	49.5 ± 1.6
ω-3	12.9 ± 2.1	15.0 ± 1.8[c]	16.6 ± 1.9[c]
ω-6	35.9 ± 1.7	33.8 ± 1.8[c]	32.9 ± 1.6[c]
ω-3/ω-6	0.36 ± 0.07	0.44 ± 0.06[c]	0.51 ± 0.07[c]

[a]Significant at $p < 0.05$,
[b]Significant at $p < 0.01$,
[c]Significant at $p < 0.001$, when compared with initial level.

TABLE 5

The Thromboxane B_2 Production in Blood Samples Obtained from Male Students on Fish Diet up to 15 Weeks and the Content of Plasma 6-keto-$PGF_{1\alpha}$ at the Same Time (means ± SD, n = number of students)

	n	Before fish diet	During fish diet (7 wk)	After fish diet (15 wk)	Decrease in 15 weeks
Production of thromboxane B_2 (nmol/100 × 10^9 platelets/l)					
Controls	13	325 ± 160	281 ± 130	279 ± 106	46 ± 108
Fish eaters	11	295 ± 134	213 ± 109	176 ± 100[a]	120 ± 118[b]
Fish eaters (reduced fat)	11	328 ± 139	255 ± 103	212 ± 129[a]	116 ± 108[c]
Plasma concentration of 6-keto-$PGF_{1\alpha}$ (ng/l)					
Controls	13	257 ± 100	252 ± 98	239 ± 106	18 ± 58
Fish eaters	13	221 ± 82	199 ± 65	177 ± 34[d]	44 ± 72
Fish eaters (reduced fat)	15	269 ± 86	254 ± 69	219 ± 55[e]	50 ± 58[f]

[a]Significant at $p < 0.01$, when compared with initial level (Wilcoxon matched-pairs, signed-ranks test).
[b]Significant at $p < 0.1$,
[c]Significant at $p < 0.05$, when compared with controls (Mann-Whitney U-test).
[d]Significant at $p < 0.05$,
[e]Significant at $p < 0.01$, when compared with initial level.
[f]Significant at $p < 0.1$, when compared with controls.

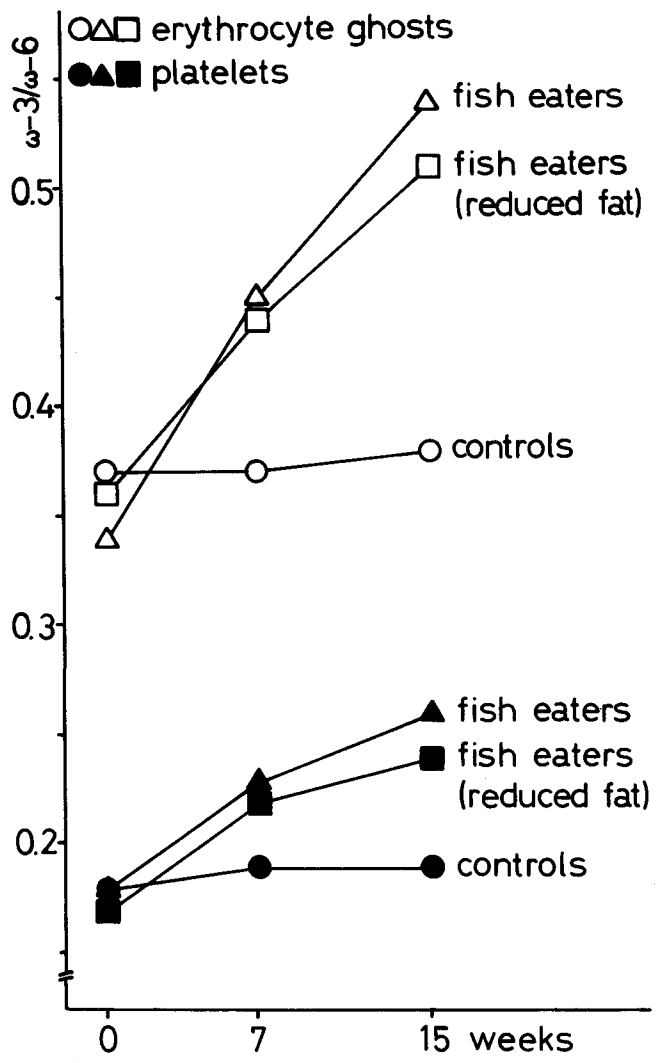

FIG. 1. The ω-3/ω-6 ratio in fatty acids of platelets and erythrocyte ghosts in male students eating an average of 3.7 weekly fish meals for 15 weeks.

acid). The changes in platelets reported by Sanders et al. (21) were somewhat greater than those in the present study. This can be explained by the shorter turnover time of platelets than of erythrocytes. Also, in the present study, the changes took place earlier in the platelets than in erythrocytes.

There are only a few controlled studies published where fish-containing meals have been added to the western diet. No similar studies have been reported with as great a number of healthy people using a normal catering system, over such a long time, as in the present study. In most of those studies, rather large amounts of marine fish have been used, and the follow-up periods have been rather short. There are, however, two previous studies in which the administration of marine fish has been comparable with our experiment. During 11 weeks (daily intake of 150–200 g fish, mainly herring, mackerel and salmon) (22) and three months (at least twice a week and at least 100 g fatty fish, i.e., 0.3–0.6 g of eicosapentaenoic acid per day) (23), a lowering of serum triglycerides was observed, although no change took place in serum cholesterol. A lowering of cholesterol has been observed when large amounts of fish have been eaten (24). In the present study, the quantity of fish corresponded to about 100 g and 1 g of ω-3 fatty acids per day. Thus, this is the smallest amount so far reported to cause a decrease in the serum triglyceride level. The dosage was, in addition, also effective in lowering apolipoprotein levels. Thorngren and coworkers (22) were unable to detect any changes in apolipoproteins in their 11-week study.

Two metabolites of arachidonic acid were measured in this study. Thromboxane B_2 is a stable hydrolyzation product of thromboxane A_2, which promotes platelet aggregation and is a vasoconstrictor. The other metabolite, 6-keto-$PGF_{1\alpha}$, is a conversion product of prostacyclin. Prostacyclin prevents aggregation and is a vasodilator.

In our study, a diminished production of thromboxane B_2 in platelets was found. Such an effect has not been previously reported with such a low intake of fish. The decrease was significant only after 15 weeks on the fish regimen. Thus, the low doses require more time to produce

significant effects. It has been reported that 1 g of eicosapentaenoic acid in capsule form, after a 10 g daily dosage, is not enough to keep the thromboxane B_2 production depressed in two patients with atherosclerosis (25). The present results indicate that already the dosage of 0.25 g per day can cause a decrease in the thromboxane B_2 formation. Although it is not possible to draw any definite conclusions, it might be more profitable to get ω-3 fatty acids in fish-containing meal than in concentrated oil form.

In the present study, a lowering of plasma 6-keto-$PGF_{1\alpha}$ was observed. It has been suggested that fish oils have little or no effect on prostacycline production in the endothelial wall (26). There is, however, one report where a lowering has been observed (24). This suggests that the fatty acid spectrum in the vascular endothelium is changing, parallel with the erythrocytes and platelets.

The students who were asked to voluntarily decrease their lipid intake showed a decrease in their serum cholesterol levels. The lowering of the lipid intake did not, however, clarify the observed differences in the fatty acid composition and prostanoid production. This suggests that the effective incorporation of ω-3 fatty acids in the membrane lipids, and subsequent changes in the prostanoid metabolism, are not necessarily dependent on the amount of other fatty acids present in the diet.

The boreal freshwater fish diet provided by public catering system, modified lipid metabolism in 15 weeks. Further deepening of the responses still could have been expected, if the diet had been continued. The results stress the importance of the diet duration when the effects of different ω-3 fatty acid doses are estimated. The results support the hypothesis of the protective effect of moderate fish consumption against coronary heart disease.

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REFERENCES

1. Sanders, T.A.B., and Roshanai, F. (1983) *Clin. Sci. 64,* 91-99.
2. Harris, W.S., Connor, W.E., and McMurry, M.P. (1983) *Metabolism 32,* 178-184.
3. Nagakawa, Y., Orimo, H., Harasawa, M., Morita, I., Yashiro, K., and Murota, S. (1983) *Atherosclerosis 47,* 71-75.
4. Siess, W., Roth, P., Scherer, B., Kurzmann, I., Böhlig, B., and Weber, P.C. (1980) *Lancet 1,* 441-444.
5. Saynor, R., Verel, D., and Gillot, T. (1984) *Atherosclerosis 50,* 3-10.
6. Kobayashi, S., Hirai, A., Terano, T., Hamazaki, T., Tamura, Y., and Kumagai, A. (1981) *Lancet ii,* 197.
7. Terano, T., Hirai, A., Hamazaki, T., Kobayashi, S., Fujita, T., Tamura, Y., and Kumagai, A. (1983) *Atherosclerosis 46,* 321-331.
8. Kromhout, D., Bosschieter, E.B., and de Lezenne Coulander, C. (1985) *N. Engl. J. Med. 312,* 1205-1209.
9. Shekelle, R.B., Missel, L.V., Oglesby, P., Shryock, A.M., and Stamler, J. (1985) *N. Engl. J. Med. 313,* 820.
10. Norell, S.E., Ahlbom, A., Feychting, M., and Pedersen, N.L. (1986) *Br. Med. J. 293,* 426.
11. Volsett, S.E., Heuch, I., and Bjelke, E. (1986) *N. Engl. J. Med. 313,* 820-821.
12. Curb, J.D., and Reed, D.M. (1986) *N. Engl. J. Med. 313,* 821.
13. Ågren, J., Muje, P., Hänninen, O., Herranen, J., and Penttilä, I. (1987) *Comp. Biochem. Physiol. 88B,* 905-909.
14. Salonen, J.T. (1982) *J. Epidem. C. 36,* 294-297.
15. Jauhiainen, M., Laitinen, M., Marniemi, J., Liippo, K., Penttilä, I., and Hietanen, E. (1983) *Clin. Chem. 29,* 1731-1735.
16. Powell, W.S. (1982) in *Methods in Enzymology* (Lands, W.E.M., and Smith, W.L., eds.) Vol. 86, pp. 467-477, Academic Press, New York, NY.
17. Bernhardt, I., Donath, E., and Glaser, R. (1984) *J. Membr. Biol. 78,* 249-255.
18. Puustinen, T., Punnonen, K., and Uotila, P. (1985) *Acta Med. Scand. 218,* 59-62.
19. Exler, J., and Weihrauch, J.L. (1976) *J. Am. Diet. Assoc. 69,* 243-248.
20. Lands, W.E.M. (1986) *Fish and Human Health,* Academic Press, New York, NY.
21. Sanders, T.A.B., Vickers, M., and Haines, A.P. (1981) *Clin. Sci. 61,* 317-324.
22. Thorngren, M., Nilsson, E., and Gustafson, A. (1986) *Acta Med. Scand. 219,* 23-28.
23. Fehily, A.M., Burr, M.L., Phillips, K.M., and Deadman, N.M. (1983) *Am. J. Clin. Nutr. 38,* 349-351.
24. Goodnight, S.H., Harris, W.S., Connor, W.E., and Illingsworth, D.R. (1982) *Arteriosclerosis 2,* 87-113.
25. Knapp, H.R., Reilly, I.A.G., Alessandrini, P., and FitzGerald, G.A. (1986) *N. Engl. J. Med. 314,* 937-942.
26. Fischer, S., and Weber, P.C. (1984) *Nature 307,* 165-168.

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Induction of Long-lasting Hypercholesterolemia in the Rat Fed a Cystine-enriched Diet

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The influence of dietary excess (5%) of L-cystine on rat plasma lipoproteins was examined. After only one week of cystine feeding, an increase in the plasma cholesterol level and a decrease in triglyceride levels were observed. The increase in cholesterol level became greater when the duration of cystine-enriched diet increased until eight weeks (+131% after eight weeks), but no further increase occurred between 8 and 20 weeks. This change was essentially due to the progressive increase in cholesterol levels in high density lipoproteins (HDL) and in lipoproteins isolated between 1.040 and 1.063 g/ml, i.e., certain low density lipoproteins (LDL_2), and containing mainly apoE-rich lipoproteins (HDL_1). The decrease in plasma triglycerides resulted from that of chylomicrons and very low density lipoproteins (VLDL). The effects observed after four or eight weeks of cystine feeding were maintained for eight weeks after replacing the cystine diet by the standard diet. Ingestion of the standard diet containing either cholestyramine (2%) or probucol (0.25%) following eight weeks of cystine feeding significantly decreased plasma cholesterol levels. It is concluded that cystine-fed rats are a useful tool of investigation for understanding mechanisms leading to increased plasma cholesterol level and for hypocholesterolemic drug trials.
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Hypercholesterolemic animals are useful models for studies concerning cholesterol homeostasis and for hypocholesterolemic drug trials. In the rat, hypercholesterolemia has generally been induced by feeding diets supplemented with cholesterol, antithyroid drugs and/or bile acids (1-6). The hyperlipoproteinemia observed was mainly characterized by an increase in very low density (VLDL) and VLDL-like particles enriched in esterified cholesterol, a pattern quite different from that observed in human hypercholesterolemia (7).

An attempt has been previously carried out to obtain hypercholesterolemic rats with amino acid-enriched diets (8). It was found that cystine supplementation (5%) was very effective. Hypercholesterolemia was due to enhanced cholesterol levels in low density lipoproteins (LDL) of density 1.040-1.063 g/ml and in high density lipoproteins (HDL) of density 1.063-1.210 g/ml, and cholesterol content in chylomicrons and VLDL (d < 1.006 g/ml) was decreased (9). These results were obtained after two months of ingestion of the cystine-enriched diet and only cholesterol and protein contents of lipoproteins were determined.

The present work was carried out in order to study how hypercholesterolemia appeared as a function of the duration of cystine-enriched-diet administration. It also was investigated whether the effect of the cystine-enriched diet on plasma lipoproteins remained unchanged after replacing this diet with the control diet. The efficacy of two hypocholesterolemic drugs, cholestyramine and probucol, was tested in rats fed the cystine-enriched diet. Plasma lipoproteins were isolated in a density gradient and analyzed for their cholesterol, triglyceride, phospholipid and protein contents. The apolipoprotein composition of some of these lipoproteins was also determined.

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Abbreviations: CETP, cholesteryl ester transfer protein; EDTA, ethylenediamine tetraacetic acid; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyl transferase; (V)LDL, (very) low density lipoprotein; MIA, monoiodoacetamide; PTU, propylthiouracil.

METHODS

Animals and diets. Male Wistar rats (from Elevage Janvier, France) weighing about 350 g were used. The standard semipurified diet (S) contained essentially sucrose 53%, casein 23% and lard 9%; the mineral mix contained copper and the cholesterol content was 0.015% (10). Rats, randomly divided into groups of four or five, were first fed the cystine-enriched diet for one to eight weeks (C groups) and then the standard diet for eight additional weeks (CS groups). A control group (n = 5) received the standard diet for 16 weeks. Two groups of rats were fed the cystine-enriched diet for four weeks, and then, for the next four weeks, the standard diet supplemented with either cholestyramine (2%) or probucol (0.25%). Blood samples (about 1 ml) were obtained at different times from the retroorbital venous sinus with heparinized capillary pipettes under light diethylether anesthesia. Plasma was separated from cells after 2 min centrifugation in an Eppendorf-type centrifuge. At the end of the experiments, blood was collected by puncture of the abdominal aorta on 4%-ethylenediamine tetraacetic acid (EDTA-4%-monoiodoacetamie (MIA) and centrifuged at 2000 *g* for 10 min at 4°C. All blood samplings were performed between 10 a.m. and noon on animals not previously starved. Cholesterol, triglyceride and phospholipids were determined in plasma samples.

Lipoprotein isolation. A density gradient (adapted from [11] was prepared in tubes with saline solutions of densities as follows: 1.5 ml, 1.006 g/ml; 1.5 ml, 1.020 g/ml; 2 ml, 1.040 g/ml; 3 ml, 1.063 g/ml; 2 ml of plasma adjusted to 1.21 g/ml by the addition of solid KBr; and 1 ml, 1.25 g/ml. The tubes were centrifuged for 24 hr at 15°C in a SW 40.1 Ti Beckman rotor at 40,000 rpm. The first 18 fractions (0.5 ml each) were collected from the meniscus of the tubes. Each fraction of lipoprotein was assayed for cholesterol content. The chemical composition of lipoproteins was determined in pooled fractions corresponding to chylomicrons and VLDL (d < 1.006 g/ml: fractions 1 and 2), LDL_1 (LDL of density between 1.006 and 1.040 g/ml: fractions 3 to 6), LDL_2 (LDL of density between 1.040 and 1.063 g/ml: fractions 7 to 11) and HDL (HDL of density between 1.063 and 1.210 g/ml: fractions 12 to 18).

Chemical analysis. Cholesterol, phospholipids and triglyceride contents were assayed using enzymatic methods

with commercial kits (Boehringer-Mannheim, Wako, TX). Proteins were determined by the method of Lowry et al. (12) using bovine serum albumin as the standard. Samples were extracted with chloroform after color development in order to avoid turbidity due to lipids.

Apolipoprotein separation. LDL_2 and HDL samples were extensively dialyzed against 0.05% EDTA, 0.9% NaCl, 0.02% NaN_3 (pH 7.4) at 4°C. The dialyzed samples were lyophilized and dissolved in Tris 0.02 M, urea 6 M, sodium dodecyl sulfate (SDS) 0.1 M, pH 8.4. A discontinuous gradient of polyacrylamide gels (5–10%) was prepared in glass tubes with added glycerol (12%), according to Connelly and Kuksis (13), in order to use non-delipidated lipoproteins. The lipoprotein samples (about 50 µl and containing 50 µg of protein, each) were mixed with isopropanol (10 µl), SDS 20% (10 µl), dithiothreitol 400 mM (10 µl), bromophenol blue 0.02% (5 µl) and glycerol (5 µl). After boiling for 3 min, the mixture was applied to the polyacrylamide gels. Electrophoresis was carried out at 1.5 mA per gel for 15 hr in a Pharmacia electrophoresis apparatus with 0.1% SDS, 0.1 M phosphate buffer solution, pH 7.0. Gels were stained with 0.25% Coomassie Brilliant Blue k-250 (in 50% methanol) and 9.2% acetic acid, and destained with 5% methanol and 7.5% acetic acid (14). Gels were scanned with an LKB 2202 Ultrascan integrator. The apolipoprotein bands were identified on the basis of molecular weights as determined by reference to purified protein standards and by comparison with published SDS gel electrophoretograms of rat apolipoproteins (6,15). All the results were expressed as means ± standard error of the mean. Significant differences were estimated either by the Student's t test or by the paired t test.

RESULTS

Body weights and food intakes. As reported in Table 1, body weights for rats at the same age were lower for those fed the cystine diet than for those fed the standard diet. This difference, however, was only significant in the C8S8 group, in which body weights were 76% of the weights in the S16 group, during the eighth week of the experiment. Food intakes observed during all the periods of feeding the standard diet, even following administration of the cystine diet, varied between 16.4 ± 0.7 (n = 5) and 20.9 ± 1.2 g/day (n = 5), without any significant differences among the various groups. However, almost all the periods of feeding the cystine diet were characterized by a food intake significantly lower than in standard animals. The greatest differences occurred during the first three weeks of cystine feeding, with a mean value of food intake of 12.1 ± 0.4 to 14.8 ± 0.7 g/day.

Plasma lipid composition. The cholesterol, triglyceride and phospholipid contents in plasma obtained from rats fed the standard diet, or the cystine-enriched diet followed by the standard diet, are reported in Table 2. Cholesterol and phospholipid contents of plasma from rats fed the standard diet did not change significantly during the 16 weeks of experiment (S16 compared with S0). The plasma-triglyceride content decreased by 32%. In rats fed the cystine-enriched diet, some changes in lipid composition of plasma appeared after only one week (C1 group): compared with C0 group, the cholesterol content increased by 45%, whereas the triglyceride content decreased by 52%. Similar changes, compared with C0 group, occurred during the following weeks of cystine feeding with progressive increases in the cholesterol amounts: 53% (C2), 87% (C4) and 131% (C8), and decreases in the triglyceride amounts: 76% (C2), 55% (C4) and 77% (C8). Except for the C2 and the C4 groups, in which the plasma-phospholipid contents were lower and higher than in control rats (−31% and +23%, respectively), no changes in this content were observed during cystine feeding. Replacement of the cystine-enriched diet by the control diet did not significantly alter plasma-cholesterol and phospholipid levels obtained with the cystine-enriched diet. However, plasma-triglyceride content was significantly increased (+71%) in the C2S8 group compared with the C2 group.

Cholesterol distribution in the lipoproteins. The cholesterol contents in the 18 lipoprotein fractions isolated by density-gradient ultracentrifugation from the plasma of rats fed only the standard diet and rats fed the cystine-enriched diet for one to eight weeks are reported in Figure 1. In control rats, plasma cholesterol was distributed as follows: 15% in fractions 1 and 2 (chylomicrons and VLDL), 3.4% in fractions 3–6 (LDL_1), 19% in fractions 7–11 (LDL_2) and 62% in fractions 12–18 (HDL). After one week of cystine feeding (C1 group), these proportions were not modified. The 48% increase of the total plasma-

TABLE 1

Body Weights (g) of Rats (5 per group) Fed the Standard Diet (S group) or the Cystine-enriched Diet Followed by the Standard Diet (CS groups)

Groups	Week number								
	0	1	2	4	8	9	10	12	16
S16	309 ± 13	344 ± 17	368 ± 19	411 ± 27	461 ± 30	475 ± 32	493 ± 32	522 ± 32	558 ± 33
C1S8	297 ± 7	304 ± 7[a]	339 ± 9	372 ± 12	411 ± 20	425 ± 20	—	—	—
C2S8	306 ± 12	321 ± 11[a]	329 ± 14[a]	382 ± 17	427 ± 23	432 ± 33	448 ± 28	—	—
C4S8	311 ± 10	318 ± 15[a]	331 ± 15[a]	348 ± 17[a]	424 ± 18	438 ± 20	451 ± 18	485 ± 20	—
C8S8	302 ± 6	309 ± 9[a]	318 ± 9[a,b]	334 ± 9[a,b]	352 ± 16[a,c]	364 ± 16[c]	386 ± 17[c]	417 ± 23[b]	456 ± 25[b]

[a]Periods of feeding the cystine-enriched diet.

[b]$P < 0.05$ and

[c]$P < 0.02$, are significant values when compared with the values from the S16 group at the same week.

Values are means ± SEM.

TABLE 2

Cholesterol, Triglyceride and Phospholipid Contents (mg/ml) of Plasma[a]

Groups	Cholesterol	Triglycerides	Phospholipids
C0	0.71 ± 0.02	2.92 ± 1.16	1.91 ± 0.05
C1	1.03 ± 0.07[b]	1.39 ± 0.19[c]	2.13 ± 0.11
C1S8	0.97 ± 0.07	2.37 ± 0.40	1.79 ± 0.12
C2	1.09 ± 0.07[d]	0.70 ± 0.05[e]	1.52 ± 0.04[d]
C2S8	1.04 ± 0.07	2.45 ± 0.45[f]	2.28 ± 0.21
C4	1.33 ± 0.07[e]	1.31 ± 0.12[e]	2.35 ± 0.06[d]
C4S8	1.18 ± 0.09	1.34 ± 0.19	2.63 ± 0.15
C8	1.64 ± 0.25[c]	0.67 ± 0.13[e]	2.57 ± 0.29
C8S8	1.32 ± 0.15	1.46 ± 0.72	2.29 ± 0.19
S0	0.82 ± 0.06	3.41 ± 0.43	2.42 ± 0.16
S16	0.99 ± 0.11	2.30 ± 0.41[f]	2.01 ± 0.15

[a]From rats fed the standard diet from the start of the experiment (C0, S0) to 16 weeks later (S16), the cystine-enriched diet for 1 to 8 weeks (C1 to C8) followed by the standard diet for 8 weeks (C1S8 to C8S8). Except for C0 group (n = 25), which is constituted by the rats of all the groups at the start of the experiment, each group contains 5 rats.

[b]$P < 0.005$,

[c]$P < 0.02$,

[d]$P < 0.01$ and

[e]$P < 0.001$, are significantly different (C1, C2, C4 and C8 groups), when compared with values in C (Student's t test).

[f]$P < 0.05$, significantly different, when values of C1S8, C2S8, C4S8 and S16 are compared wth those of C1, C2, C4, C8 and S0 groups (paired t test), respectively.

Mean values ± SEM.

cholesterol content was due to a slight increase in all of the lipoprotein fractions, but mainly in fractions 11–15 (density 1.063 to 1.150 g/ml). After two weeks of cystine feeding, a decrease in plasma cholesterol carried by chylomicrons and VLDL, and an increase in that carried by LDL_2, became apparent. The two measurements represented 7% and 27%, respectively, of the total plasma-cholesterol content. These proportions were 4% and 30% after four weeks of cystine feeding, and 2% and 41% after eight weeks. At this time, the marked increase (131%) in the plasma-cholesterol level, compared with rats fed the standard diet, essentially was due to an increase in LDL_2 and HDL cholesterol. The most marked increase was observed in fraction 10 (d: 1.058 g/ml): 395%.

Figure 2 shows the distribution of cholesterol among 18 lipoprotein fractions isolated from rats fed the cystine diet for four weeks, and from rats fed the cystine diet for four weeks and then the standard diet for either four or eight weeks. In comparison with the profile obtained after four weeks of cystine feeding, no major changes occurred after replacing this diet by the standard one for four or eight weeks. A slight increase was observed in LDL_2 after four weeks, and a slight decrease occurred in HDL at four or eight weeks. Similar observations were made when rats were fed the cystine diet for eight weeks followed by the standard diet.

Lipoprotein composition. The chemical composition of lipoproteins (proteins, cholesterol, triglycerides and phospholipids expressed as percentage of the total lipoprotein weight) from rats fed the standard diet (C0 group), or the cystine-enriched diet for four weeks (C4 group) or eight weeks (C8 group), followed by the standard diet for eight weeks (C4S8 and C8S8 groups), are reported in Table 3.

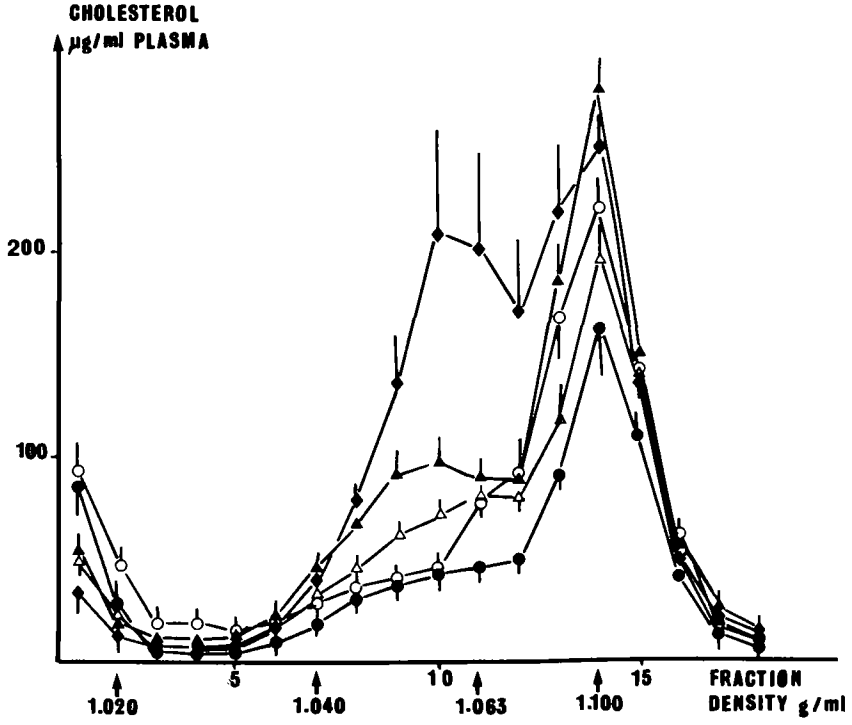

FIG. 1. Cholesterol distribution in 18 lipoprotein fractions from rats fed the standard diet (●) or the cystine-enriched diet for 1 (○), 2 (△), 4 (▲) or 8 (◆) weeks (n = 5 for each group, means ± SEM).

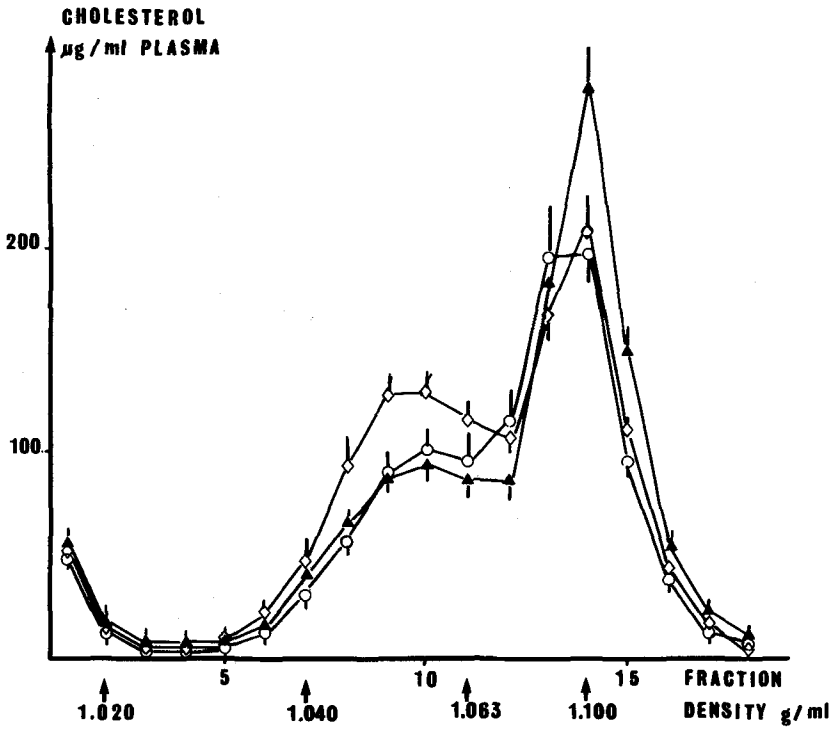

FIG. 2. Cholesterol distribution in 18 lipoprotein fractions from rats fed a cystine-enriched diet for 4 weeks (▲) or for 4 weeks followed by 4 (◊) or 8 (○) weeks of standard diet (n = 5 for each group, means ± SEM).

TABLE 3

Protein, Cholesterol, Triglyceride and Phospholipid Content (as Percentage of Total Lipoprotein) in Lipoproteins[a]

Constitutent	Groups	Chylomicrons + VLDL	LDL_1	LDL_2	HDL
Proteins	C0	7.5 ± 0.5	17.0 ± 1.2	35.4 ± 2.0	56.5 ± 0.9
	C4	9.7 ± 0.4[c]	19.6 ± 1.8	27.2 ± 0.7[d]	50.0 ± 1.1[d]
	C4S8	8.6 ± 0.8	17.9 ± 1.9	30.5 ± 0.6[b,h]	49.0 ± 0.2[d]
	C8	12.8 ± 1.9[b]	24.6 ± 1.0[d]	31.7	53.6 ± 1.4
	C8S8	12.2 ± 1.4[b]	24.0 ± 2.2	34.1 ± 0.5	53.8 ± 0.8
Cholesterol	C0	2.8 ± 0.3	7.0 ± 2.7	27.7 ± 1.6	16.5 ± 2.1
	C4	5.6 ± 0.4[d]	26.2 ± 3.9[c]	34.0 ± 0.7[d]	17.7 ± 0.3
	C4S8	4.4 ± 0.3[c]	13.5 ± 1.3[b,f]	29.2 ± 0.9[h]	16.1 ± 0.5[f]
	C8	11.1 ± 2.5[b]	24.2 ± 3.6[c]	34.2	18.9 ± 0.6
	C8S8	7.5 ± 1.4[b]	27.2 ± 4.4[c]	33.1 ± 1.1[b]	18.2 ± 0.5
Triglycerides	C0	75.0 ± 1.5	58.2 ± 6.5	12.5 ± 2.5	2.7 ± 0.7
	C4	71.3 ± 1.3	31.5 ± 6.6[b]	1.8 ± 0.3[b]	1.1 ± 0.1
	C4S8	71.2 ± 1.3	45.5 ± 2.7	1.1 ± 0.5[b]	1.4 ± 0.2
	C8	54.5 ± 5.4[c]	33.7 ± 4.0[c]	3.0	3.8 ± 0.6
	C8S8	67.1 ± 4.1	28.3 ± 8.1[b]	2.5 ± 1.0[d]	1.3 ± 0.3[g]
Phospholipids	C0	14.6 ± 1.0	17.8 ± 0.3	24.4 ± 2.0	26.7 ± 1.3
	C4	13.3 ± 0.5	22.7 ± 1.9	37.0 ± 1.1[e]	31.2 ± 1.0[b]
	C4S8	15.8 ± 0.7[f]	23.1 ± 0.8	39.1 ± 0.8[e]	33.4 ± 0.4[c]
	C8	15.9 ± 1.9	17.5 ± 1.3	31.0	23.7 ± 1.3
	C8S8	12.9 ± 1.3	20.4 ± 1.7	30.3 ± 0.7[b]	26.7 ± 0.5

[a]Isolated from rats fed the standard diet (C0), or the cystine-enriched diet for 4 (C4) or 8 weeks (C8) followed by the standard diet for 8 weeks (C4S8 and C8S8).

[b]$P < 0.05$,

[c]$P < 0.02$,

[d]$P < 0.01$ and

[e]$P < 0.001$ (Student's t test), are significantly different than those values obtained in the C0 group.

[f]$P < 0.05$ and

[g]$P < 0.01$ (paired t test), are values significantly different in the C4S8 group compared with the C4 group, or in the C8S8 group compared with the C8 group.

Means ± SEM (n = 5, for each group).

TABLE 4

Apoprotein Composition (as Percentage of Total Apoproteins) of LDL_2 and HDL[a]

	Groups	$ApoB_{100}$	$ApoB_{48}$	$ApoA_{IV}$	ApoE	$ApoA_I$	ApoC
LDL_2	S16	4 ± 2	1	—	34 ± 1	21 ± 1	40 ± 2
	C8	13 ± 4	3	1	46 ± 3[c]	20 ± 3	16 ± 2[d]
	C8S8	2 ± 1	1	1	37 ± 4	33 ± 3[c]	28 ± 4[b]
HDL	S16	—	—	12 ± 1	3 ± 1	56 ± 3	29 ± 1
	C8	—	—	11 ± 1	10 ± 2[b]	53 ± 3	26 ± 1
	C8S8	—	—	8 ± 1	1 ± 1	61 ± 4	29 ± 2

[a]From rats (3–4 per group) fed the standard diet for 16 weeks (S16), or rats fed the cystine-enriched diet for 8 weeks (C8) followed by the standard diet for 8 weeks (C8S8).

[b]$P < 0.02$,

[c]$P < 0.01$ and

[d]$P < 0.001$, are significantly different than those values obtained in the S16 group.

Means ± SEM.

The major changes occurring after feeding the cystine-enriched diet were an increase in the relative proportion of cholesterol in the lighest lipoproteins, chylomicrons and VLDL (+100% after four weeks and +296% after eight weeks) and LDL_1 (+274% after four weeks and +246% after eight weeks). A decrease in the triglyceride proportion was shown for LDL_1 and LDL_2 after four weeks of cystine feeding (−46% and −86%, respectively) and appeared in chylomicrons and VLDL after eight weeks (−27%). After replacing the cystine-enriched diet with the standard diet, the cholesterol enrichment persisted in chylomicrons plus VLDL and in LDL_1 (+57% and +93%, respectively, for the C4S8 group; and +168% and +288%, respectively, for the C8S8 group). The decrease in the triglyceride proportion of LDL_2 remained quite unchanged (−76%).

Apolipoproteins. The apolipoprotein composition (expressed as percentage of total apolipoproteins) of LDL_2 and HDL from rats fed the standard diet for 16 weeks (S16), and rats fed the cystine-enriched diet for eight weeks (C8) followed by the standard diet for eight weeks (C8S8), is reported in Table 4. An enrichment in apoE was observed in LDL_2 and HDL after eight weeks of cystine feeding (34% and 233%, respectively). In LDL_2, this enrichment was associated with a decrease in the apoC proportion (by (60%). Eight weeks after replacement of the cystine diet with the standard diet, the apoE proportion in LDL_2 and HDL returned to a value close to that of control rats (S16), whereas in LDL_2, the apoC proportion still remained slightly lower than in control rats and the $apoA_I$ proportion was slightly higher.

Drug trials. Plasma cholesterol was significantly decreased by probucol and cholestyramine (29% and 28%, respectively), in rats previously fed a cystine-enriched diet (Table 5). Cholesterol concentrations were decreased in LDL_2 and HDL and the values for HDL returned to the levels observed in control rats. However, LDL_2-cholesterol concentrations still remained higher than in control rats.

TABLE 5

Cholesterol Levels (μg/ml) in Plasma, LDL_2 and HDL of Rats[a]

Groups	Plasma	LDL_2	HDL
C0	801 ± 63	153 ± 39	501 ± 29
C4S4	1320 ± 70	532 ± 26	663 ± 49
C4Pro	940 ± 50[c]	323 ± 49[c,d]	539 ± 32
C4Cy	950 ± 50[c]	305 ± 40[c,d]	490 ± 31[b]

[a]Fed either the standard diet (C0) or the cystine-enriched diet for 4 weeks, and then either the standard diet (C4S4) alone or the standard diet supplemented with probucol (0.25%, C4Pro) or cholestyramine (2%, C4Cy) for 4 weeks.

[b]$P < 0.02$ and

[c]$P < 0.01$, are significantly different in the C Pro and C Cy groups than in the C S group.

[d]$P < 0.05$, is significantly different in the C Pro and C Cy groups than in the C group.

Means ± SEM (n = 5).

DISCUSSION

Studies carried out in man and in experimental animals have clearly shown that the plasma-cholesterol level can be influenced by the nature of dietary proteins (16,17). In the rat (18–20) and in hypercholesterolemic subjects (21–23), plasma cholesterol was lower with soy protein than with casein diet. The amino acid composition of these proteins has been implicated in that effect (17,18,24,25). It has been reported, in various species, that some individual amino acids added in excess into diets increase the plasma cholesterol levels, whereas others decrease it or have no effect. It has been observed that, among six amino acids used in excess in the diet, only cystine (5% added) increases the rat plasma cholesterol level (8). Therefore, a new model of hypercholesterolemia of endogenous origin was available. This model brought forth new information concerning the regulation of plasma cholesterol (26).

From the first week onwards, the cystine-enriched diet reduced body weight increase and food intake, as previously described (8), increased plasma-cholesterol level and decreased plasma-triglyceride levels. These above changes were amplified with the duration of cystine ingestion to reach maximal effect after eight weeks

(+ 131% in cholesterol and −77% in triglycerides). No further increase in plasma cholesterol occurred when the cystine diet was given for a longer time. (At 20 weeks, plasma cholesterol was equal to 1.32 ± 0.20 mg/ml.) Cessation of the cystine diet did not induce a quick return to a normal plasma-cholesterol level. Hypercholesterolemia persisted for eight weeks after replacement of the cystine diet (given previously for four weeks) with the standard diet (Fig. 2). It persisted for 28 weeks after replacing the cystine diet given for 20 weeks with the standard diet (1.40 ± 0.20 mg/ml). It is interesting that, in Wistar rats fed the semipurified diet (sucrose-rich and containing lard) ad libitum, the plasma-triglyceride level is very high when animals are not fasted before blood sampling.

In contrast to human plasma cholesterol that is carried mainly by LDL ($1.019 < d < 1.063$) and contains only apoB, the rat plasma cholesterol is carried essentially by HDL ($1.063 < d < 1.21$). The rat lipoproteins of density range 1.019–1.063 are constituted by two fractions as shown by ultracentrifugation in a zonal system or on an isopycnic gradient—the lightest is apoB-rich (LDL) and the heaviest (HDL_1) is apoE-rich (27). In the present study, LDL of density range 1.006–1.063 have been separated into 2 fractions—LDL_1 ($1.006 < d < 1.040$) and LDL_2 ($1.040 < d < 1.063$), according to Fidge and Poulis (28). LDL_1 contain mainly apoB (over 70% of total proteins, results not shown), whereas LDL_2 contain apoE, $apoA_I$ and apoC as major apolipoproteins and a minor amount of apoB. In this study, although HDL_1 (or apoE-rich HDL) had not been isolated by affinity chromatography (29) or electrophoresis (30), the low apoB and high apoE contents of LDL_2 suggest that this lipoprotein fraction is largely constituted of HDL_1. The low apoE and high $apoA_I$ contents in HDL ($1.063 < d < 1.21$) reflect that these HDL are mainly $apoA_I$-rich HDL, or HDL_2. Nevertheless, taking into account the absolute amounts of proteins in lipoproteins and the relative proportions of the different apolipoproteins, it can be estimated that the absolute amount of apoE in LDL_2 is similar to that in HDL (about 50 μg/ml plasma), but the amount of $apoA_I$ is about 20 times greater in HDL (about 800 μg/ml plasma), than in LDL_2 (about 40 μg/ml). Therefore, assuming that the apoE content could reflect the presence of HDL_1, similar amounts of HDL_1 seemed to be present in the 2 fractions, LDL_2 and HDL. Therefore, HDL_1 would be the major lipoprotein contained in LDL_2, but only a minor one in HDL.

The characteristics of hypercholesterolemia from endogenous origin, obtained in rats fed a cystine-enriched diet, are opposite to those of hypercholesterolemia from exogenous origin, obtained in rats fed a cholesterol-enriched diet. As compared with normal rats, in rats fed the cystine-enriched diet for eight weeks, cholesterol distribution in lipoproteins is characterized by a decrease in chylomicrons + VLDL (fractions 1 + 2 in the density gradient), an increase in LDL_2 (fractions 7–11), that particularly affects fraction 10 (+395%), and an increase in HDL (fractions 12–18). In cholesterol-fed rats, high chylomicrons + VLDL levels and low LDL_2 and HDL levels were reported (9). Whereas, in control rats with cholesterol essentially being carried by HDL (63%) and LDL_2 (20%), eight weeks of cystine feeding enhances the participation of LDL_2 in the cholesterol transport (41%). The hypercholesterolemia with the relatively high LDL_2- and HDL-cholesterol content of the cystine diet, which was given for 4 or 8 weeks, persisted for eight more weeks after replacement with the normal diet. Cessation of the cystine diet did not induce a quick return to a normal situation, because 40% of the plasma cholesterol is still transported by LDL_2 (C8S8).

Some changes in the chemical composition of lipoproteins were observed from the fourth week of cystine feeding (Table 3). The relative proportions of cholesterol in chylomicrons + VLDL, LDL_1 and LDL_2 were increased, and those of triglycerides in LDL_1 and LDL_2 (C4) in chylomicrons + VLDL, LDL_1 and LDL_2 (C8) were decreased. Replacement of the cystine diet with the standard diet was not sufficient to recover a normal chemical composition of these lipoproteins, which remained cholesterol-enriched and triglyceride-poor. Both the low triglyceride proportion and the low triglyceride levels in the lighest lipoproteins certainly resulted from a very active lipolysis.

The plasma apoE content in LDL_2 and in HDL was similarly increased (fivefold) in cystine-fed rats, resulting again in identical apoE amounts in these two lipoprotein fractions. The $apoA_I$ content was multipled by three in LDL_2 and by 1.5 in HDL, and the apoB content in LDL_2 was 10-fold that of control rats. The increase in apoE-rich lipoproteins could result from an increased apoE production. The increase in apoB-rich LDL could be explained by either an increased production or a delayed catabolism, because apoE and apoB compete for binding to the apoB,E receptor (31). After eight weeks of cystine feeding, cessation of the cystine diet for eight more weeks decreased the absolute amount of apoE in HDL, which returned to control values, but that of LDL_2 remained high.

In the rat, the plasma HDL_1 level depends on the nutritional or experimental conditions. It is increased by diets enriched with cholesterol and propylthiouracil (PTU), which is an antithyroid drug (2,5), PTU alone (32), cystine excess (9), and by copper-deficient diet (33). The plasma HDL_1 level decreases with a cholesterol-enriched diet, without addition of PTU (5,9), and with essential fatty acid deficiency (34). It can be noted that apoE-rich HDL, normally not present in human plasma, have been observed in some pathological disorders as primary biliary cirrhosis (35), familial lecithin:cholesterol acyl transferase (LCAT) deficiency (36) and alcoholic hepatitis (37).

The origin of HDL_1 is now largely documented, although all the mechanisms involved in production of this lipoprotein are not fully understood. First, it was suggested that HDL_1 could originate as a product of the catabolism of chylomicrons (27). Now it is well known that lipolysis of the triglyceride-rich lipoproteins provides surface constituents (free cholesterol, phospholipids and apolipoproteins) in the HDL density range (HDL_3 and very high density lipoproteins or VHDL) (38,39). It also was demonstrated that the conversion of HDL_3 to HDL_2, and then to HDL_1, occurs with free cholesterol- and apoE-enrichment and esterification of free cholesterol by LCAT (30,31,40). In species devoid of cholesteryl ester transfer protein (CETP), such as the rat (15), the conversion is complete. In the presence of CETP, the conversion is stopped at HDL_2, because esterified cholesterol

can be transported from HDL_2 to VLDL, with the remaining lipoprotein returning to the HDL_3 density range (40). This explains why, under normal conditions, rat plasma, in contrast to human plasma, contains HDL_1. Our own results with control, cholesterol-fed and cystine-fed rats (26) show a strong correlation between liver-cholesterol synthesis and the plasma LDL_2 (rich in HDL_1) level, and suggest that cholesterol necessary for the formation of HDL_1 can be provided by the liver, perhaps in synergy with apoE production. Other recent experiments, not yet published, suggest that the hepatic lipase, which is involved in the lipolysis of triglyceride-rich lipoproteins, play an important role in the appearance of apoE-rich HDL_1.

Because rats fed a cystine-enriched diet developed a relatively long-lasting hypercholesterolemia, the effects of two drugs known to be hypocholesterolemic were investigated. Results showed that both drugs were effective in this model of hypercholesterolemia. HDL-cholesterol levels returned to normal values, but LDL_2-cholesterol levels were not fully normalized by the drugs at the dosage used.

In conclusion, rats fed a cystine-enriched diet constitute, even after cessation of this particular diet, a new tool for investigating the mechanisms that increase plasma-cholesterol and, in particular, apoE-rich-HDL_1 levels.

REFERENCES

1. Lasser, N.L., Roheim, P.S., Edelstein, D., and Eder, H.A. (1973) *J. Lipid Res. 14,* 1-8.
2. Mahley, R.W., and Holcombe, K.S. (1977) *J. Lipid Res. 18,* 314-324.
3. Noel, S.P., Wong, L., Dolphin, P.J., Dory, L., and Rubinstein, D. (1979) *J. Clin. Invest. 64,* 674-683.
4. Swift, L.L., Manowitz, N.R., Dunn, G.D., and Lequire, V.S. (1980) *J. Clin. Invest. 66,* 415-425.
5. Delamatre, J.G., and Roheim, P.S. (1981) *J. Lipid Res. 22,* 297-306.
6. Dolphin, P.J. (1981) *J. Lipid Res. 22,* 971-989.
7. Goldstein, J.L., and Brown, M.S. (1983) in *The Metabolic Basis of Inherited Disease* (Standbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L., and Brown, M.S., eds.) pp. 672-712, McGraw-Hill Book Company, New York, NY.
8. Sérougne, C., and Rukaj, A. (1983) *Ann. Nutr. Metab. 27,* 386-395.
9. Sérougne, C., Férézou, J., and Rukaj, A. (1984) *Ann. Nutr. Metab. 28,* 311-320.
10. Mathé, D., Lutton, C., Rautureau, J., Coste, T., Gouffier, E., Sulpice, J.-C., and Chevallier, F. (1977) *J. Nutr. 107,* 466-474.
11. Terpstra, A.H.M., Woodward, C.J.H., and Sanchez-Muniz, F.J. (1981) *Anal. Biochem. 111,* 149-157.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
13. Connelly, P.W., and Kuksis, A. (1982) *Biochim. Biophys. Acta 711,* 245-251.
14. Weber, K., and Osborn, M. (1969) *J. Biol. Chem. 244,* 4406-4412.
15. Oschry, Y., and Eisenberg, S. (1982) *J. Lipid Res. 23,* 1099-1106.
16. Carroll, K.K. (1978) *Nutr. Rev. 36,* 1-5.
17. Kritchevsky, D. (1979) *J. Am. Oil Chem. Soc. 56,* 135-140.
18. Yadav, N.R., and Liener, I.E. (1977) *Nutr. Rep. Int. 16,* 385-389.
19. Nagata, Y., Tanaka, K., and Sugano, M. (1981) *Br. J. Nutr. 45,* 233-241.
20. Terpstra, A.H.M., Van Tintelen, G., and West, C.E. (1982) *J. Nutr. 112,* 810-817.
21. Sirtori, C.R., Gatti, E., Mantero, O., Conti, F., Agradi, E., Tremoli, E., Sirtori, M., Fraterrigo, L., Tavazzi, L., and Kritchevsky, D. (1979) *Am. J. Clin. Nutr. 32,* 1645-1658.
22. Wolfe, B., Giovanetti, P., Cheng, D.C.H., Roberts, D.C.K., and Carroll, K.K. (1981) *Nutr. Rep. Int. 24,* 1187-1198.
23. Goldberg, A.P., Lin, A., Kolar, J.B., Grundhauser, J.J., Steinke, F.H., and Schonfeld, G. (1982) *Atherosclerosis 43,* 355-368.
24. Huff, M.W., Hamilton, R.M.G., and Carroll, K.K. (1977) *Atherosclerosis 28,* 187-195.
25. Sugano, M. (1983) in *Animal and Vegetable Proteins in Lipid Metabolism and Atherosclerosis* (Gibney, M.J., and Kritchevsky, D., eds.) pp. 51-81, Alan R. Liss Inc., New York, NY.
26. Sérougne, C., Férézou, J., and Rukaj, A. (1987) *Biochim. Biophys. Acta 921,* 522-530.
27. Lusk, L.T., Walker, L.F., DuBien, L.H., and Getz, G.S. (1979) *Biochem. J. 183,* 83-90.
28. Fidge, N.H., and Poulis, P. (1978) *J. Lipid Res. 19,* 342-349.
29. Weisgraber, K.H., and Mahley, R.W. (1980) *J. Lipid Res. 21,* 316-325.
30. Gordon, V., Innerarity, T.L., and Mahley, R.W. (1983) *J. Biol. Chem. 258,* 6202-6212.
31. Koo, C., Innerarity, T.L., and Mahley, R.W. (1985) *J. Biol. Chem. 260,* 11934-11943.
32. Dory, L., and Roheim, P.S. (1981) *J. Lipid Res. 22,* 287-296.
33. Lefevre, M., Keen, C.L., Lönnerdal, B., Herley, L.S., and Schneeman, B.O. (1986) *J. Nutr. 116,* 1735-1746.
34. Lowe, K.E., Pelkey, S., Williams, M.A., and Nichols, A.V. (1988) *Lipids 23,* 106-114.
35. Koga, S., Miyata, Y., Ibayashi, H. (1985) *Hepatology 5,* 286-292.
36. Mitchell, C.D., King, W.C., Applegate, K.R., Forte, T., Glomset, J.A., Norum, K.R., and Gjone, E. (1980) *J. Lipid Res. 21,* 625-634.
37. Weidman, S.W., Ragland, J.B., and Sabesin, S.M. (1982) *J. Lipid Res. 23,* 556-569.
38. Tall, A.R., and Small, J.M. (1978) *New Engl. J. Med. 299,* 1232-1236.
39. Eisenberg, S. (1984) *J. Lipid Res. 25,* 1017-1058.
40. Gavish, D., Oschry, Y., and Eisenberg, S. (1987) *J. Lipid Res. 28,* 257-267.

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Hydrolysis of a Fluorescent Substance Formed From an Oxidized Phospholipid and an Amino Compound by Phospholipase A_2

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Phosphatidylcholine hydroperoxide produced a fluorescent substance (FS-III) through reaction with 1-aminopentane after preincubation with heme methyl ester as a model system. The FS-III was retained at the 2-position of the glycerol backbone of phosphatidylcholine without breakdown into low molecular weight compounds. Phosphatidylcholine oxidized by catalysis with ferrous ion and ascorbic acid also produced the same fluorescent substance (FS-III). Phospholipase A_2 specifically hydrolyzed the FS-III attached to the phospholipid, making it possible to elute the same fluorescent substance (FS-II) as that obtained from oxidized methyl linoleate. The release of FS-II by hydrolysis of FS-III attached to phospholipid increased with greater phospholipase A_2 activity. It is suggested that, with aging, the accumulation of fluorescent lipofuscin pigments in biomembranes may be related to changes in the peroxidized phospholipid content and that phospholipase A_2 may play a role in decreasing the formation and accumulation of fluorescent phospholipids in biomembranes.
Lipids 23, 937-941 (1988).

The degradative compounds derived from hydroperoxides (HPO) of polyunsaturated fatty acids (PUFA) in the presence of metals, ascorbic acid or heme methyl ester (heme) compounds are involved in fluorescence formation through reaction with amino compounds in vitro (1,2). These fluorescent substances exhibit fluorescence spectra with excitation and emission maxima at 320-350 nm and 410-430 nm, respectively. These are very similar to the fluorescence spectra of fluorescent lipofuscin pigments, associated with aging, in animal tissues (3-5).

We recently have reported that both the 9- and the 13-HPO of methyl linoleate (ML) produced fluorescent substances exhibiting the same fluorescence spectra through reaction with 1-aminopentane (1-AP) as a model amino compound after preincubation with heme (6). PUFA are acylated mainly at the 2-glycerol position of phospholipid and are converted to HPO without release from phospholipid at the early stage of lipid peroxidation (7,8). For example, linoleic acid attached to phospholipid produces 9- and 13-HPO derivatives without fatty acid release (8). HPO of 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC) also showed similar fluorescence characteristics through reaction with 1-AP in the presence of heme (9). Moreover, the fluorescent substance was attached to PLPC and was released from the glycerol backbone after transmethylation. These results suggest that acylated fatty acid peroxides in phospholipids may play an important role in the formation of fluorescent substances with various amino compounds in biomembranes.

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Abbreviations: 1-AP, 1-aminopentane; heme, heme methyl ester(s); HPO, hydroperoxide(s); ML, methyl linoleate; PLPC, 1-palmitoyl-2-linoleoyl-phosphatidylcholine; PUFA, polyunsaturated fatty acid(s); HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

PUFA moieties attached to the 2-glycerol position of phospholipids generally can be hydrolyzed with phospholipase A_2. It recently was reported that phospholipase A_2 also preferentially hydrolyzes peroxidized fatty acid esters in phospholipids (10-12). In the present study, therefore, we investigated the phospholipase A_2-dependent hydrolysis of the fluorescent substances attached to the 2-position of the glycerol backbone of phosphatidylcholine. The role of phospholipase A_2 in the accumulation of fluorescent lipofuscin pigments in biomembranes is discussed.

MATERIALS AND METHODS

Materials. Linoleic acid, ML, PLPC, hemin (type I), soybean lipoxygenase (Type I) and phospholipase A_2 (from porcine pancreas, 650 units/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO). 1-AP and 5% methanolic *m*-trifluoromethylphenyltrimethyl ammonium hydroxide were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Heme was prepared according to the method of Ortiz de Montellano et al. (13).

Preparation of ML-HPO, PLPC-HPO and oxidized ML or PLPC. ML-HPO and PLPC-HPO were prepared using lipoxygenase, according to the methods previously reported (9). Direct oxidation of ML and PLPC by iron and ascorbate was carried out by the following procedure. To ML or PLPC (1 μmol each) dispersed with 0.2 ml of 0.1% Tween 80 solution by vortexing, 1.4 ml of 0.1 M Tris buffer (pH 7.4), 0.2 ml of 0.1 mM $FeSO_4$ and 0.2 ml of 1 mM ascorbic acid were added, and this mixture was incubated at 37°C for 3 hr. Oxidized ML or PLPC was extracted with 5 ml of a chloroform/methanol (2:1) mixture and the chloroform layer was evaporated under an N_2 stream.

Formation of fluorescent substances. Fluorescence formation through reaction of ML-HPO or PLPC-HPO (100 nmol) with 1-AP (1 μmol) in the presence of heme (1 nmol) was carried out, according to the method previously reported (6,9). Oxidized ML or PLPC was dissolved in 0.2 ml of chloroform, and 1.8 ml of methanol was added. This solution was preincubated with or without heme (1 nmol) at 37°C for 30 min. One ml of 1-AP (1 μmol) in methanol was added to each respective preincubation mixture and the mixture was further incubated, at 37°C for 20 hr, for fluorescence formation. Fluorscence spectra were measured with a Hitachi MPF-3 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) and fluorescence intensity was expressed as a percentage of that intensity of a quinine sulfate standard (0.1 μg/ml in 0.1 N H_2SO_4). The total fluorescence intensity of each sample was adjusted to the same fluorescence strength (700%) by concentration under an N_2 stream, and an aliquot of each was subjected to high performance liquid chromatography (HPLC). HPLC was performed on a μ-Bondapak FAA column (3.9 × 300 mm, Waters Associates, Milford, MA), using methanol/water (8:2) for the first 10 min, followed by methanol/water (9:1) as the mobile phases at a flow rate of 1.0 ml/min. A packed guard column (4 ×

50 mm, Unisil C_{18}, 10 μm, Gasukuro Kogyo Inc., Tokyo, Japan) was used. Fluorescence peaks were monitored at excitation and emission maxima, 350 nm and 420 nm, with a Hitachi 650-10LC fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). The fluorescent substances were then subjected to thin layer chromatography (TLC) (Kieselgel 60, Merck, Darmstadt, FRG) and developed with chloroform/methanol (9:1).

Hydrolysis of fluorescent substances with phospholipase A_2. Fifty μl of each fluorescent sample were evaporated under an N_2 stream and were dispersed in 0.2 ml of 10 mM sodium deoxycholate by vortexing. The reaction was carried out at 37°C for 30 min in 2 ml of 0.1 M Tris buffer (pH 8.4), containing 5 mM $CaCl_2$ and 13 units of phospholipase A_2. Reaction products were extracted with 5 ml of chloroform/methanol (2:1) and methylated with diazomethane in ethyl ether at 25°C for 15 min.

RESULTS

Purified ML-HPO or PLPC-HPO and oxidized ML or PLPC were separately incubated with 1-AP in methanol solution at 37°C for 20 hr after preincubation with or without heme. Both ML-HPO and PLPC-HPO produced fluorescent substances exhibiting the same fluorescence spectra with excitation and emission maxima at 340–350 nm and 410–425 nm, respectively, while those HPO incubated without heme produced only a small number of fluorescent substances (Table 1). On the other hand, both oxidized ML and PLPC directly produced fluorescent substances having the same fluorescence spectra, through reaction with 1-AP in the absence of heme. Moreover, these fluorescence intensities showed a twofold increase, after preincubation with heme, accompanied by degradation of their HPO.

We previously reported that fluorescent substances formed from secondary oxidation products of ML with 1-AP in methanol solution revealed two fluorescence peaks (FS-I and FS-II) upon reverse-phase HPLC (14). Similarly, oxidized ML also produced two fluorescent substances, FS-I and FS-II, by reaction with 1-AP, as shown in Figure 1 B, and ML-HPO revealed one major fluorescence peak (FS-II) and one small peak (FS-I). On the other hand, when fluorescent substances formed from PLPC-HPO with 1-AP after decomposition by heme were subjected to reverse-phase HPLC, a new major fluorescence peak (FS-III) was eluted at a retention time of 18.5 min, but only small peaks of FS-I and FS-II were detected (Fig. 1A). However, oxidized PLPC produced the new fluorescence peak (FS-III) and the FS-I peak, but not FS-II. It could be inferred from these results that FS-I was formed from both oxidized ML and PLPC, but not from the degraded compounds produced from ML-HPO and PLPC-HPO by heme.

The fluorescent substances formed from both PLPC-HPO and oxidized PLPC were transmethylated with *m*-trifluoromethylphenyltrimethyl ammonium hydroxide in methanol solution according to the method previously described (9). After transmethylation, the FS-II peak appeared at a retention time of 8.5 min, but the FS-III peak disappeared (Fig. 2A). The appearance of the FS-II peak after transmethylation supports the possibility that the new major fluorescent substance (FS-III) is retained at the 2-position of glycerol backbone in PLPC.

Phospholipase A_2 preferentially hydrolyzes PUFA esters at the 2-position of phospholipids. Therefore, the possible role of phospholipase A_2 in the release of fluorescent substances formed from oxidized phospholipids also was examined in this study. When the fluorescent substances formed from PLPC-HPO were incubated with

TABLE 1

Fluorescence Formation from Oxidized PLPC or ML and their HPO with 1-AP

	heme	Excitation maximum (nm)	Emission maximum (nm)	Fluorescence intensity (%)
PLPC-HPO	−	348	418	21.6
	+	348	420	114.2
ML-HPO	−	348	418	28.0
	+	350	418	140.0
Oxidized PLPC	−	350	418	125.8
	+	350	420	226.0
Oxidized ML	−	348	418	147.3
	+	350	420	276.7

Both PLPC- and ML-HPO (100 nmol) were separately preincubated with or without heme (1 nmol) in 2 ml of methanol at 37°C for 30 min. One μmol of PLPC or ML oxidized with Fe^{++} and ascorbate also was preincubated with or without heme (1 nmol) under the same conditions. One ml of 1-AP (1 μmol) in methanol was added to the each preincubation mixture and then incubated at 37°C for 20 hr for fluorescence formation.

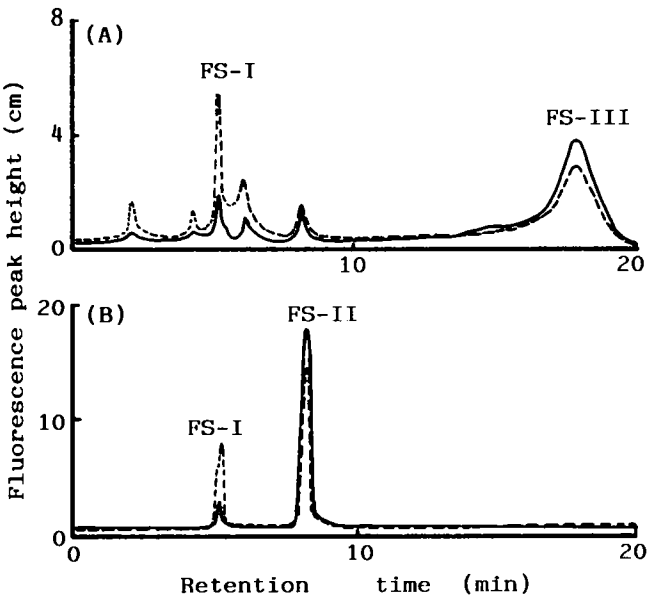

FIG. 1. HPLC of fluorescent substances formed from oxidized PLPC or ML and their HPO with 1-AP. Reverse-phase HPLC was performed on a μ-Bondapak FAA and elution was carried out with methanol/water (80:20 and 90:10) mixtures at a flow rate of 1 ml/min Aliquots of fluorescent solution formed from PLPC-HPO (A, ——), oxidized-PLPC (A, – – –), ML-HPO (B, ——) or oxidized-ML (B, – – –) with 1-AP in the presence of heme were subjected to HPLC using the methods described in Materials and Methods.

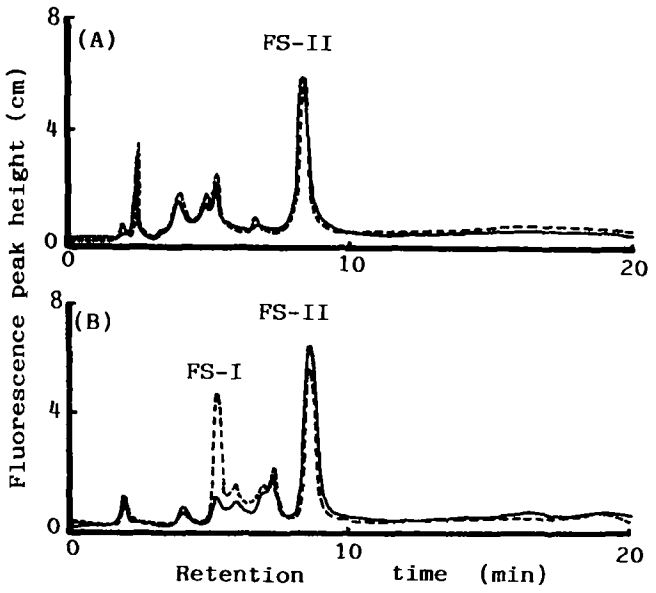

FIG. 2. HPLC of fluorescent substances formed from PLPC-HPO or oxidized PLPC with 1-AP after transmethylation and phospholipase A_2 hydrolysis. HPLC was performed as described in Fig. 1. Fluorescent substances formed from both PLPC-HPO (—) and oxidized PLPC (– – –) were transmethylated (A) or hydrolyzed with phospholipase A_2 (B) as described in Materials and Methods.

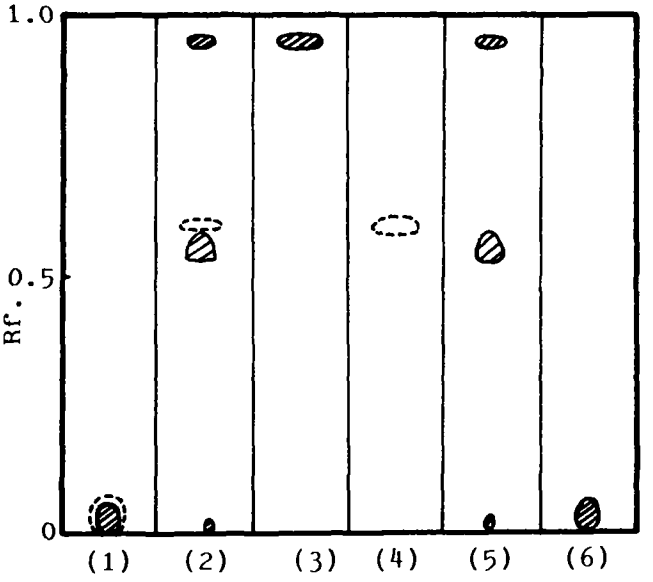

FIG. 3. TLC of fluorescent substances formed from ML-HPO or PLPC-HPO with 1-AP. Aliquots of fluorescent solution formed from PLPC-HPO were subjected to TLC before (1) and after (2) phospholipase A_2 hydrolysis. ML (3), fluorescent substances (4) formed from ML-HPO and PLPC incubated with (5) and without (6) phospholipase A_2 were also subjected to TLC. Fluorescent spots (broken oval) were detected by excitation at around 360 nm, while other spots (crosshatched oval) were detected under iodine vapor.

phospholipase A_2 and methylated with diazomethane, the FS-III peak disappeared completely and the FS-II peak appeared at a retention time of 8.5 min in HPLC (Fig. 2B). Similarly, the FS-III formed from oxidized PLPC also changed to the FS-II, following the same treatment as described above, but the FS-I peak was not influenced by hydrolysis with phospholipase A_2 and eluted at the same retention time.

A diagram of the TLC plates is shown in Figure 3. The fluorescent substance (FS-II) formed from ML-HPO showed an R_f of 0.60, and the FS-III spot formed from PLPC-HPO was detected at the origin. After treatment of FS-III with phospholipase A_2, the fluorescent substance was detected at the same R_f value (0.60) as that of FS-II.

When FS-I and FS-III formed from oxidized PLPC were collected by HPLC and separately incubated with phospholipase A_2, FS-III specifically produced FS-II and the level of FS-II released from hydrolysis of FS-III increased with greater phospholipase A_2 activity (Fig. 4), but FS-I did not appear to be influenced by phospholipase A_2.

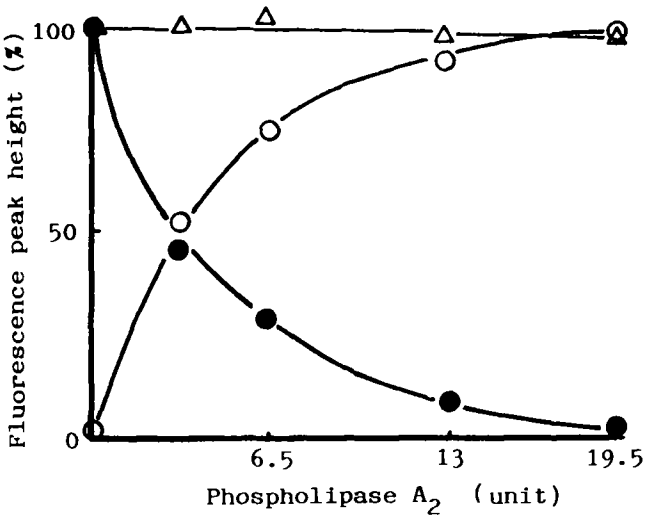

FIG. 4. Hydrolysis of FS-III with phospholipase A_2 and appearance of FS-II. FS-I and FS-III formed from oxidized PLPC with 1-AP were purified by HPLC. An aliquot of each fluorescent substance was hydrolyzed with phospholipase A_2 and methylated with diazomethane. After that, each fluorescence peak height of FS-I (△), FS-II (○) and FS-III (●) was measured on HPLC using the method as described in Materials and Methods. Fluorescence intensities were expressed as the percentage of a nontreated sample.

DISCUSSION

Fluorescent lipofuscin pigments associated with aging are believed to be formed from lipid peroxides with amino compounds in animal tissues (4,5). Although malondialdehyde reacts with amino compounds to form fluorescent substances in vitro (15,16), malondialdehyde has not yet been conclusively identified in biological tissues. Therefore, the role of malondialdehyde in the in vivo formation of lipofuscin pigments seems to have been exaggerated (4).

On the other hand, there are numerous reports that HPO are easily degraded by many types of catalysts such as metals, ascorbic acid or heme compounds (17–19), and that the degradative compounds produced are involved in the formation of fluorescent substances through reaction with amino compounds (1,2,20). For example,

through autoxidation, ML produces 9- and 13-positional monohydroperoxide isomers having the *cis-trans* and *trans-trans* configurations, and, in the presence of heme, all of them produce fluorescent substances with the excitation and emission maxima at 350 nm and 420 nm, respectively, by reaction with amino compound (6). Decomposition of monohydroperoxides of methyl linolenate forms dimeric or monomeric compounds and many kinds of volatile breakdown products including monoaldehydes such as propanal, 2-pentenal or 2,4-heptadienal (21). Although these monoaldehydes also produce fluorescent substances through reaction with amino compounds in vitro (22,23), Frankel et al. (24) have reported that monomeric compounds, other than these volatile aldehydes prepared from methyl linolenate HPO, produce a higher level of fluorescence. We also have demonstrated that secondary oxidation products prepared from autoxidized ML are directly involved in the formation of fluorescent substances (14).

PUFA in biological tissues are mainly localized in membrane phospholipids and are converted to HPO without release from their glycerol backbone (7,8). Miyazawa et al. (25) recently succeeded in confirming the presence of phosphatidylcholine HPO in normal human blood. These results seem to imply that phospholipid HPO are involved in the formation of fluorescent substances in biological tissues. As a model system, when PLPC-HPO was incubated with 1-AP after decomposition by heme, PLPC-HPO also produced a fluorescent substance having the same fluorescence spectrum as that obtained from ML-HPO, which was attached to PLPC without being released from its glycerol backbone (9). Nielsen (26) also reported that peroxidized cardiolipin produced a fluorescent substance through reaction with albumin. Moreover, Shimasaki et al. (27) have reported that the fluorescent substances formed from oxidized liposomes containing rat liver phosphatidylcholine and phosphatidylethanolamine remain in the liposome membranes. The results obtained in the present study also strongly suggest that peroxidized PUFA substituted at the 2-glycerol position in phospholipid are directly involved in the formation of fluorescent substances through reaction with amino compounds without hydrolysis.

It is well known that phospholipase A_2 release PUFA from the 2-glycerol position in membrane phospholipids. Moreover, it has been shown recently that membrane-associated phospholipase A_2 displayed significantly greater activity against acylated fatty acid peroxides in phospholipids (10-12). The elimination of peroxidized fatty acids from membrane phospholipids was shown to protect membranes from peroxidation damage, and then, phospholipase A_2 was necessary for the detoxification of the lipid peroxides (12). Moreover, Jung et al. (28) reported that phosphatidylcholine, oxygenated at the carbon-15 position of the arachidonic acid moiety by 15-lipoxygenase in human polymorphonuclear leukocytes, was hydrolyzed by phospholipase A_2 at a faster rate than nonoxygenated phosphatidylcholine. Ozawa et al. (29) also suggested that phospholipase A_2 played a key role in releasing leukotoxin in lung lavages from rats.

Based on these reports of the preferential hydrolysis of peroxidized phospholipids with phospholipase A_2, we now provide additional support for the concept of phospholipase A_2 having an important role in the regulation of fluorescent substances as they accumulate as end products of lipid peroxidation and cause membrane damage. As revealed by HPLC and shown in Figure 2B, FS-III attached to PLPC was hydrolyzed with phospholipase A_2 and released the resulting fluorescent substance at the same retention time (8.5 min) as that of FS-II formed from ML-HPO. These results suggest that the fluorescent substance (FS-III) formed from oxidized phospholipid is retained at the 2-glycerol position, and that the fluorescent moiety may be gradually released from phospholipid by hydrolysis with phospholipase A_2. On the other hand, FS-I formed from both oxidized ML and PLPC with 1-AP may be derived from breakdown products (e.g., reactive carbonyls) of linoleic acid by iron and ascorbate, because FS-I was not affected with phospholipase A_2.

Lipid peroxides are derived mainly from PUFA attached to phospholipids of biomembranes, and Palmer et al. (30) have reported that liver lipopigments in sheep contain substantial quantities of phospholipid and linoleate. This result also indicates that peroxidized phospholipids may be involved in the formation of fluorescent chromolipids in lipofuscin pigments. Moreover, it is suggested that, with aging, the accumulation of fluorescent lipofuscin pigments in biomembranes may be related to the changes occurring in peroxidized phospholipid content, and phospholipase A_2 may play a role in controlling the formation and accumulation of fluorescent lipofuscin pigments in animal tissues. Kuijk et al. (11) have discussed the novel role of phospholipase deficiency as a causal factor in degenerative diseases such as neuronal ceroid lipofuscinosis.

REFERENCES

1. Fujimoto, K., Neff, W.E., and Frankel, E.N. (1984) *Biochim. Biophys. Acta 795,* 100-107.
2. Gutteridge, J.M.C. (1985) *Biochim. Biophys. Acta 834,* 144-148.
3. Manwaring, J.D., and Csallany, A.S. (1981) *J. Nutr. 111,* 2172-2179.
4. Kikugawa, K. (1986) *Adv. Free Rad. Biol. Med. 2,* 389-417.
5. Tsuchida, M., Miura, T., and Aibara, K. (1987) *Chem. Phys. Lipids 44,* 297-325.
6. Iio, T., Yoden, K., and Tabata, T. (1987) *Chem. Pharm. Bull. 35,* 5015-5019.
7. Porter, N.A. (1984) in *Methods in Enzymology* (Packer, L., and Board, A., eds.) Vol. 105, 273-282, Academic Press, New York, NY.
8. Porter, N.A., and Wagner, C.R. (1986) *Adv. Free Rad. Biol. Med. 2,* 283-323.
9. Iio, T., and Yoden, K. (1988) *Lipids 23,* 65-67.
10. Sevanian, A., Stein, R.A., and Mead, J.F. (1981) *Lipids 16,* 781-789.
11. van Kuijk, F.J.G.M., Handelman, G.J., and Dratz, E.A. (1985) *J. Free Rad. Biol. Med. 1,* 421-427.
12. Vrsini, F., Bonaldo, L., Maiorino, M., and Gregolin, C. (1983) *J. Chromatogr. 270,* 301-308.
13. Ortiz de Montellano, R.P., and Mico, B.A. (1980) *Mol. Pharmacol. 18,* 128-135.
14. Iio, T., and Yoden, K. (1987) *Life Sci. 40,* 2297-2302.
15. Kikugawa, K., Maruyama, T., Machida, Y., and Kurechi, T. (1981) *Chem. Pharm. Bull. 29,* 1423-1432.
16. Kikugawa, K., Machida, Y., Kida, M., and Kurechi, T. (1981) *Chem. Pharm. Bull. 29,* 3003-3011.
17. Gardner, H.W. (1987) in *Autoxidation of Unsaturated Lipids* (Chan, H.W.-S., ed.) pp. 51-93, Academic Press, London, England.
18. Hamberg, M. (1983) *Biochim. Biophys. Acta 752,* 191-197.
19. Dix, T.A., and Marnett, L.J. (1985) *J. Biol. Chem. 260,* 5351-5357.

20. Shimasaki, H., and Privett, O.S. (1977) *J. Am. Oil Chem. Soc. 54,* 119-123.
21. Frankel, E.N., Neff, W.E., Selke, E., and Brooks, D.D. (1987) *Lipids 22,* 322-327.
22. Kikugawa, K., and Sawamura, A. (1987) *J. Am. Oil Chem. Soc. 64,* 1156-1162.
23. Hasegawa, K., Fujimoto, K., Kaneda, T., and Frankel, E.N. (1987) *Lipid Peroxide Res. 11,* 82.
24. Frankel, E.N., Neff, W.E., Brooks, D.D., and Fujimoto, K. (1987) *Biochim. Biophys. Acta 919,* 239-244.
25. Miyazawa, T., Yasuda, K., and Fujimoto, K. (1987) *Anal. Lett. 20,* 915-925.
26. Nielsen, H. (1978) *Lipids 13,* 253-258.
27. Shimasaki, H., Ueta, N. Mowri, H., and Inoue, K. (1984) *Biochim. Biophys. Acta 792,* 123-129.
28. Jung, G.J., Yang, D.C., and Nakao, A. (1985) *Biochem. Biophys. Res. Commun. 130,* 559-566.
29. Ozawa, T., Hayakawa, M., Takamura, T., Sugiyama, S., Suzuki, K., Iwata, M., Taki, F., and Tomita, T. (1986) *Biochem. Biophys. Res. Commun. 134,* 1071-1078.
30. Palmer, D.N., Husbands, D.R., Winter, P.J., Blunt, J.W., and Jolly, R.D. (1986) *J. Biol. Chem. 261,* 1766-1772.

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Arachidonic Acid Uptake by Phospholipids and Triacylglycerols of Rat Brain Subcellular Membranes

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In the presence of ATP, $MgCl_2$ and CoASH, different subcellular membrane fractions isolated from rat cerebral cortex exhibited characteristic profiles for the incorporation of [1-^{14}C]arachidonic acid into phospholipids and triacylglycerols. In general, uptake of label by phosphatidylcholines was higher in the synaptic membranes, and that by phosphatidylinositols was higher in the microsomes and somal plasma membranes. A substantial amount of the labeled arachidonate was also incorporated into triacylglycerols, especially in the somal plasma membranes and microsomes. Enzymes mediating the transfer of arachidonic acid to phospholipids were unstable with respect to sample storage and exposure to elevated temperatures. In contrast, the acyltransferase for triacylglycerols was more stable to these factors. Washing the membranes with bovine serum albumin resulted in an enhancement of the incorporation of label into phosphatidylinositols without affecting that of phosphatidylcholines, but the incorporation into triacylglycerols was inhibited. Treatment of synaptosomes and plasma membranes with saponin resulted in an enhancement in the labeling of phospholipids, but the labeling of triacylglycerols was inhibited. Thus, although labeled arachidonic acid was incorporated into phospholipids and triacylglycerols in brain subcellular membranes, these two types of acyltransferases exhibited different properties and responded differently to membrane perturbing agents. *Lipids 23,* **942-947 (1988).**

Not only are the phospholipids in brain important in providing the structural stability of neuronal membranes, they also are actively engaged in metabolic activities essential for maintaining the proper functioning of the membranes. A characteristic feature of this metabolism is the presence of enzymes for the deacylation-reacylation of phosphoglycerides, through which acyl groups may be modified (1). In brain membranes, arachidonic acid is specifically involved in deacylation-reacylation activity (2), and active uptake by phosphatidylinositols (PI) and phosphatidylcholines (PC) has been reported (3). More recent studies have shown that each of these acyltransfer processes is catalyzed by a separate acyltransferase (4). There is also evidence that fatty acid ligase is present in most subcellular membranes in conjunction with acyltransferases, so that fatty acids can be effectively activated and transferred to the lysophospholipid within the same membrane domain (5).

Baker and Chang (6) reported that the uptake of fatty acids by triacylglycerols (TG) is a specially active process in neuronal nuclear membranes. Furthermore, the diacylglycerol (DG) acyltransferase in these membranes showed a high selectivity for arachidonoyl-CoA (7). In neuroblastoma cells, the active uptake of arachidonic acid by TG is regarded as an important cellular mechanism for conserving fatty acids in the culture medium for the biosynthesis of phospholipids (8,9).

Our previous studies with respect to arachidonic acid metabolism in brain subcellular membranes have been confined mainly to examining its incorporation into phospholipids (3,10,11). In view of the increasing attention to understanding the arachidonic acid metabolism among brain phospholipids as well as TG, the main objective for this study is to examine these transfer processes among different brain subcellular membrane fractions. In addition, some factors that may differentially alter the uptake of arachidonic acid by phospholipids and TG also are described.

MATERIALS AND METHODS

[1-^{14}C]Arachidonic acid (specific radioactivity 54.9 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA). ATP, dithiothreitol, $MgCl_2$, saponin and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). Organic solvents were high performance liquid chromatographic (HPLC) grade obtained either from Taylor Chemical Co. (St. Louis, MO) or Fisher Scientific (St. Louis, MO).

Animals and brain subcellular fractionation. Male Sprague-Dawley rats weighing 100-200 g were originally purchased from Taconics Farm, Inc. (Germantown, NY) and were maintained at the Sinclair Comparative Medicine Resarch Farm facilities until time of experiment. Rats were killed by decapitation, after which the cerebral cortices were quickly removed and homogenized in 20 vol of 0.32 M sucrose with 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA. Brain tissue was homogenized in seven up-and-down strokes using a glass Potter-Evehjem type homogenizer with Teflon pestle.

Brain homogenate was subjected to a series of differential and sucrose gradient centrifugation steps, according to the recently improved procedure as described by Sun et al. (12). Part of this procedure had incorporated the method of Booth and Clark (13) for the separation of myelin, synaptosomes and mitochondria fractions by sucrose-Ficoll gradient centrifugation. Furthermore, the procedure also included the isolation of a somal plasma membrane (PM) fraction by centrifugation of the post-mitochondrial supernatant at 48,000 × *g* for 20 min. This membrane fraction has been extensively characterized and was found to contain marker enzymes different than microsomes and synaptic plasma membranes (SPM) (12). The final membrane pellets resulting from the fractionation procedure were suspended in 0.32 M sucrose with 50 mM Tris-HCl (pH 7.4). An aliquot of the suspension was routinely taken for protein determination, according

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Abbreviations: BSA, bovine serum albumin; DG, diacylglycerol(s); PC, phosphatidylcholine(s); PI, phosphatidylinositol(s); PM, somal plasma membrane(s); SPM, synaptic plasma membrane(s); TG, triacylglycerol(s); HPLC, high performance liquid chromatograph(ic,y); TLC, thin layer chromatograph(ic,y).

to the procedure of Lowry et al. (14), using BSA as the standard.

Assay of arachidonate uptake activity. The incorporation of [1-^{14}C]arachidonic acid into membrane lipids was evaluated by incubation of membranes (200 μg protein) in the presence of [1-^{14}C]arachidonic acid (0.1 μCi), CoASH (0.1 mM), dithiothreitol (0.3 mM), ATP (2.5 mM), $MgCl_2$ (10 mM) and 50 mM Tris-HCl (pH 7.4) in a total volume of 1 ml. The incubation was carried out at 37°C for 10 min. Because this assay procedure was designed to examine the uptake of labeled arachidonic acid by various phospholipids and TG among different subcellular membrane fractions, no exogenous lysophospholipids or cold arachidonic acid was added to the incubation system.

Lipid extraction and analysis. The reaction was terminated by adding 4 vol of chloroform/methanol (2:1, v/v) followed by vigorous mixing and centrifugation to separate the aqueous and organic layers. The organic layer was removed and the upper phase was further extracted by 2 vol of chloroform/methanol/12 N HCl (4:1:0.013, v/v/v). The acidified organic extract was removed and neutralized with 4 N NH_4OH (1 drop) before combining with the first organic extract. After evaporation of the organic solvents under nitrogen, the samples were redissolved in chloroform/methanol (2:1, v/v) and kept at −20°C until further use.

Separation of phospholipids as well as neutral lipids was carried out by thin layer chromatography (TLC), as described by Sun (15). Initially, a portion of the lipid extract was spotted on 20 × 20 cm Silica Gel G TLC plates (250 μ thick, Analtech, Newark, DE). The solvent system for development of the TLC plates in the first dimension was chloroform/methanol/18 M NH_4OH (135:60:10, v/v/v). After development, the lipid area at the solvent front was removed and transferred to test tubes. The TLC plates were then exposed to concentrated HCl fumes for 3 min to hydrolyze the alkenylether bond of plasmalogens (16). After removing the excess HCl fumes from the plate, by blowing with an air gun, the TLC plates were developed in the second dimension with chloroform/methanol/acetone/acetic acid/0.1 M ammonium acetate (140:60:55:3.5:10, v/v/v/v/v). The lipid spots were visualized by exposing the TLC plates to iodine vapor. They were subsequently scraped and transferred to scintillation vials containing 10 ml of a toluene-base cocktail (Taylor Chemical Co.). Radioactivity was measured by a Beckman LS5800 scintillation spectrometer (Beckman Instrument, Inc., Fullerton, CA).

The neutral lipids from the solvent front area of the TLC plate were separated from the Silica Gel G by eluting with chloroform/methanol (5:1, v/v). The lipids were concentrated and applied to TLC plates that were developed in a solvent system containing hexane/diethylether/acetic acid (85:15:2, v/v/v). Because brain membranes contain very small amounts of these neutral lipids (except for cholesterol), appropriate standards were added in order to identify the lipid bands. Lipid bands were visualized through exposure to iodine vapor and were similarly transferred into scintillation vials for the counting of radioactivity.

RESULTS

Using the procedure described by Sun et al. (12), brain homogenate was fractionated into eight membrane fractions. The purity of these subcellular fractions has been assessed previously by assays of marker enzymes, analysis of their lipid profiles, as well as ultrastructural examination under electron microscope (12). In order to compare the extent of arachidonic acid transfer to various phospholipids and TG among the subcellular fractions, the protein concentration of each membrane fraction was adjusted to 2 mg/ml and 200 μg of protein was taken for each assay. When these subcellular membranes were

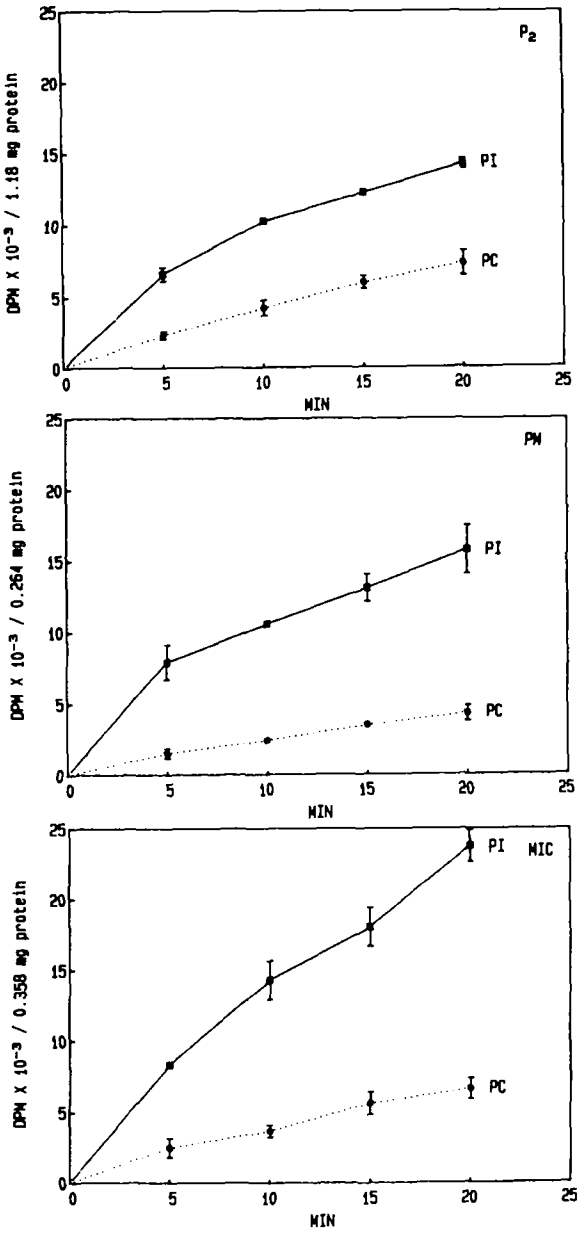

FIG. 1. Time course for the incorporation of [1-^{14}C]arachidonic acid into PI and PC of brain subcellular fractions. The incubation mixture contained: [1-^{14}C]arachidonic acid (0.1 μCi), ATP (2.5 mM), $MgCl_2$ (10 mM), CoASH (0.1 mM), membranes (protein indicated) and Tris-HCl buffer (pH 7.4) in a total volume of 1 ml. Incubation of samples was carried out in triplicates at 37°C for 10 min. The reactions were terminated by adding 4 vol of chloroform/methanol (2:1, v/v) and lipids were extracted as described in the text. Radioactivity of the lipids (DPM) is expressed as the means ± SD from triplicate determinations.

incubated with [1-^{14}C]arachidonic acid in the presence of ATP, $MgCl_2$ and CoASH, radioactivity was incorporated mainly into PI, PC and TG. Under this incubation condition, labeled arachidonic acid was most actively utilized by microsomes that corresponded to 35% within a 10 min incubation time. Examination of the uptake pattern with time indicated a steady increase in labeling of the phospholipids among all three subcellular fractions examined (Fig. 1).

Different subcellular membranes showed different profiles in the distribution of radioactivity among PC, PI and TG (Fig. 2). In this regard, the uptake of arachidonate by PC was enhanced in the synaptic fractions (i.e., synaptosomes, SPM and synaptic vesicles) and was very low in myelin (Fig. 2a). On the other hand, the uptake of arachidonate by PI was highest in microsomes followed by PM (Fig. 2b). Similar to the labeling of PI, the uptake by TG was highest in PM followed by microsomal fractions (Fig. 2c). These results indicate that brain subcellular membrane fractions are different in their ability to take up arachidonic acid.

Because a considerably high level of radioactivity was incorporated into the glycerolipids of PM, an experiment was carried out to examine the pH profile for these acyltransfer processes. As shown in Figure 3, the transfer of arachidonate to PC and PI showed a similar pH profile that was more active towards the alkaline pH range. On the other hand, the transfer to TG showed a more restricted pH profile with peak activity between 7 and 7.5.

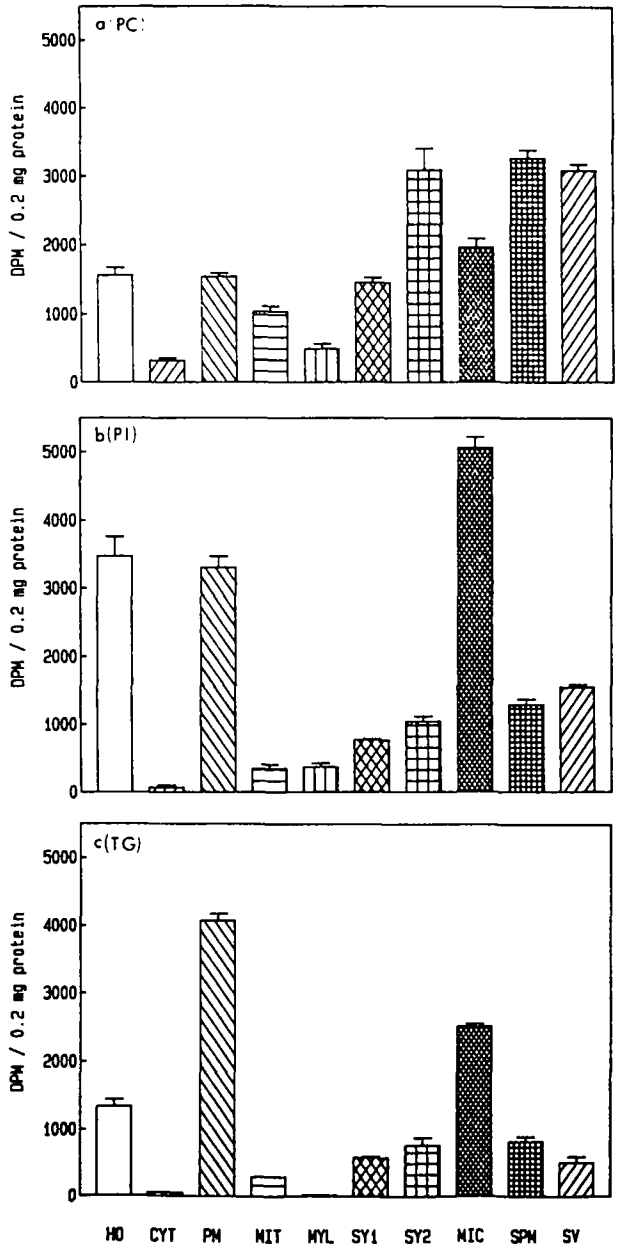

FIG. 2. A comparison of arachidonate uptake by PC (a), PI (b) and TG (c) among different brain subcellular fractions. Brain homogenate was subjected to the subcellular fractionation procedure as described by Sun et al. (12). Incubation of subcellular membranes was the same as described in Fig. 1, except that 0.2 mg of protein was used for each fraction and incubation was carried out at 37°C for 10 min. Radioactivity of the lipids (DPM) are expressed as means ± SD from triplicate determinations. Similar patterns were obtained in two different preparations. HO: homogenate. CYT: cytosol. MIT: mitochondria. MYL: myelin. SY1: intact synaptosomes. SY2: synaptosomes after shock. MIC: microsomes. SV: synaptic vesicles.

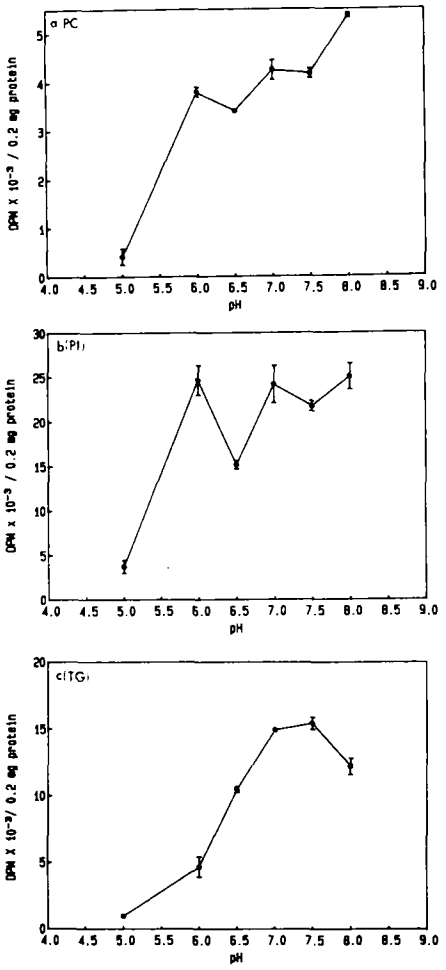

FIG. 3. The pH profile of arachidonate uptake by PC (a), PI (b) and TG (c) in PM fractions. The incubation procedure was the same as described in Fig. 1. Sodium acetate buffer was used for assays in the pH 5-6 range and Tris-HCl buffer was used for pH 7-8.

Results in Figure 4 show the effects of BSA (1 mg/ml) on labeling of the glycerolipids in PM. In agreement with the results from our previous study (10), BSA increased the labeling of PI without affecting that of PC. In addition, results here also show that BSA inhibited the transfer of labeled arachidonic acid to TG. In order to test for enzyme stability, PM were preincubated at 37°C for different time periods prior to assay of the arachidonate incorporation activity. The data in Figure 5 show a decline in labeling of both phospholipids on preincubation of membranes at 37°C. Furthermore, the decline in labeling of PI was more rapid than that of PC. In another study, PM were assayed for arachidonate uptake activity immediately after isolation (ca. 2 hr after homogenization of brain tissue) and after storage of sample at 4°C for 19 hr. Results indicated that the labeling of TG was not affected by sample storage although a substantial decrease in the labeling of phospholipids was observed (Fig. 6).

Because synaptosomes are pinched off nerve ending particles, the low acyltransferase activity in this fraction may be due to a limitation of ATP and CoASH to interact with enzymes present in the intrasynaptic sites. Frequently, saponin is used to facilitate permeability of cell plasma membranes by negatively charged compounds. Consequently, an experiment was designed to test whether pretreatment of synaptosomes with saponin (0.1%) would enhance the acyltransfer process. In addition, testing the effect of saponin with the PM fraction would allow us to discern whether its action on the acyltransferase is due to membrane permeability or due to its presence as a detergent. As shown in Figure 7, both subcellular fractions indicated an increase in arachidonoyl transfer activity to PI and PC after saponin treatment. On the other hand, saponin treatment resulted in a decrease in the labeling of TG.

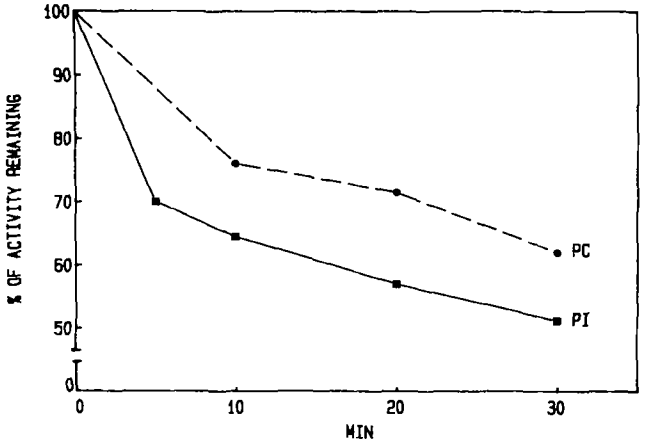

FIG. 4. The effect of BSA (1 mg/ml) on arachidonate incorporation into PM lipids. Conditions for incubation of PM was the same as that described in Fig. 1. Results are presented as the means ± SD from triplicate determinations.

DISCUSSION

In this study, we have taken advantage of our recently improved procedure for the isolation of brain subcellular fractions (12) to further investigate the ability of these membrane fractions to activate arachidonic acid and transfer it to various glycerolipids. With the exception of myelin, most membrane fractions were able to activate the arachidonic acid to its acyl-CoA in the presence of ATP, $MgCl_2$ and CoASH, and transfer the acyl-CoA to phospholipids and TG. In agreement with our earlier data (3), labeled arachidonic acid was preferentially transferred to PI and PC in most brain subcellular fractions. In addition, data from this study showed that arachidonic acid was also incorporated into TG. Because no exogenous lysophospholipids were added to the incubation mixture, it is reasonable to conclude that the uptake activity by different subcellular membranes reflects both activity of

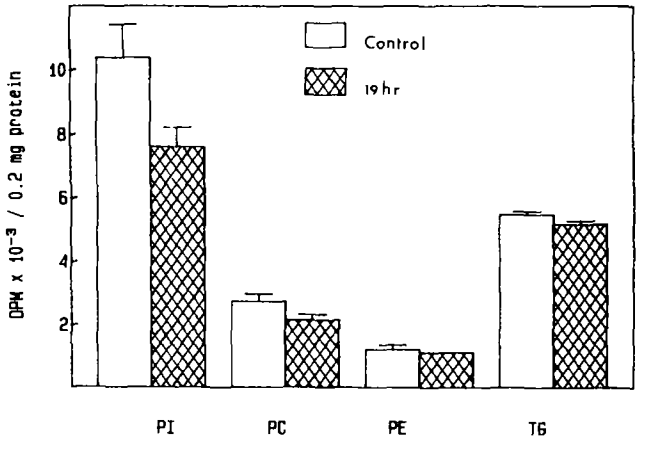

FIG. 5. Effect of preincubation on arachidonoyl transfer activity. Synaptosomes were preincubated at 37°C for the times specified, during which aliquots were taken for assay of the arachidonic acid uptake activity according to procedure described in Fig. 1. Values are the mean from triplicate determinations expressed as percentage of control.

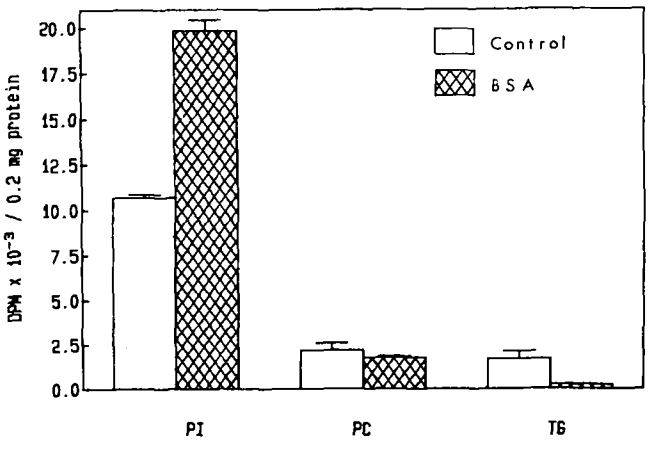

FIG. 6. The effect of sample storage on arachidonoyl transfer activity in the PM fraction. After isolation (ca. 2 hr), the PM fraction was suspended in buffer and placed in an ice bath for 0 and 19 hr. Aliquots were taken for measurement of the arachidonoyl transfer activity according to the procedure described in Fig. 1. Values are the mean ± SD of radioactivity in individual lipids obtained from triplicate determinations.

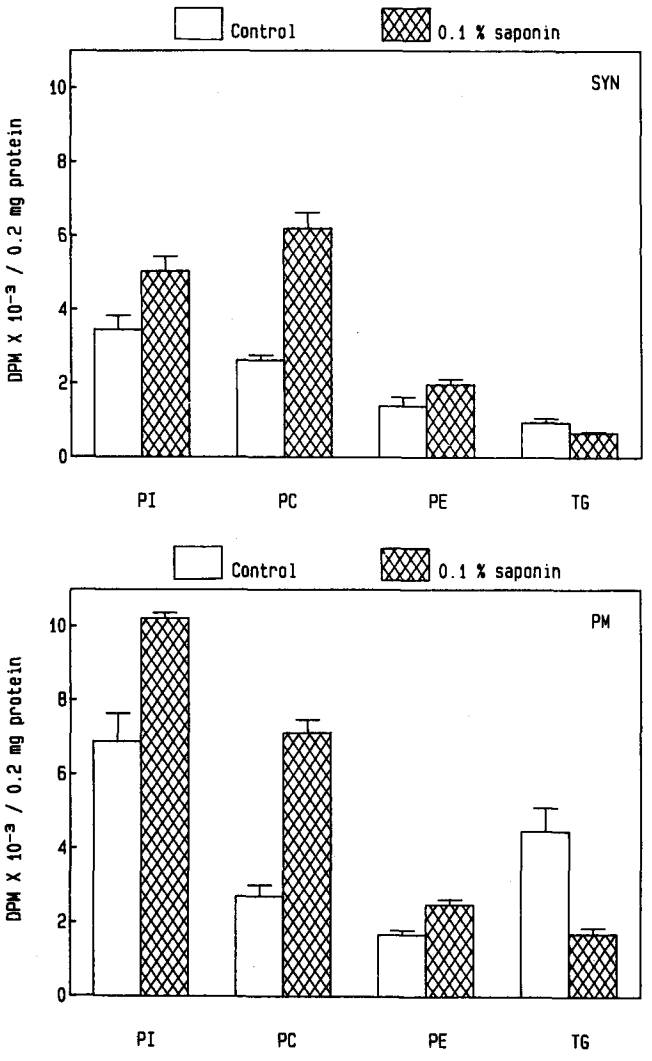

FIG. 7. The effect of saponin (0.1%) pretreatment on arachidonate incorporation into phospholipids and TG in synaptosomes and PM. Incubation condition was the same as described in Fig. 1, except that saponin was added to membranes prior to assay of the uptake activity. Values are the mean ± SD of radioactivity in PC, PI and TG obtained from triplicate determinations.

the acyltransferases and the level of endogenous lysophospholipids and DG in the membrane.

Different subcellular membrane fractions exhibit different uptake profiles for the transfer of arachidonic acid to PI, PC and TG. Of special interest is the active transfer of arachidonoyl group to PI and TG in PM and the differences in the labeling of phospholipids and TG between PM and SPM. Since the PM fraction is likely to include a large portion of plasma membranes from glial cells, it is tempting to conclude that the synthesis of TG through DG acyltransferase may be a characteristic property of glial plasma membranes. This process may be important in transporting and remodeling the TG from blood prior to entering the brain cells.

It is well recognized that the fatty acid profile of TG in brain is quite different from that in blood plasma. Fatty acids of TG in brain are enriched with C20:4 and C22:6, but lacking the C18:2 (17,18), whereas the TG in blood are high in C18:2, but contain little C20:4 and C22:6. Although TG in brain is maintained at a very low level, its unique acyl group profile suggests that the molecules must be engaged in some specific functions in brain. In addition to the PM fraction, high DG acyltransferase activity was also reported in neuronal nuclei membranes (6,7). In cultured neuroblastoma and glioma cells, labeled fatty acids added to the culture medium are readily taken up by phospholipids as well as TG (8,9). There is further evidence that the active uptake of fatty acids by TG in these cells is important for consolidating fatty acids for phospholipid biosynthesis.

The PI in brain is enriched in stearic and arachidonic acids (18), and this acyl group profile is probably attained through an active deacylation-reacylation mechanism (1). This modification is probably most active in microsomes and PM. Because most previous studies on this acyltransfer process were carried out with the crude microsomal fraction in which PM were included, information regarding the ability of PM to remodel phospholipids through the deacylation-reacylation mechanism has not been well substantiated. In a recent study with cultured neuroblastoma cells, plasma membranes isolated from these cells also showed the ability to remodel phospholipids through the deacylation-reacylation cycle as well as the de novo mechanism (19).

It is interesting to find that labeled arachidonate was more actively incorporated into PC than PI in the synaptic membrane fractions (SPM and synaptic vesicles). The exact physiological significance for this labeling pattern is not known. It is possible that these membranes contain a higher endogenous level of lysophosphatidylcholines that resulted in greater acylation to PC. Because these membranes have limited ability to synthesize phospholipids through the de novo route, it is not surprising that an active deacylation-reacylation mechanism is present in these membranes for generation of a metabolically active PC pool for synaptic function.

Ahtough acyltransferases are, in general, sensitive to membrane perturbing agents (11), uptake of arachidonate by membrane phospholipids and TG is also dependent on other factors such as the endogenous free arachidonate levels, acyl-CoA ligase activity and the acyl acceptor levels. Results here indicate that uptake of labeled arachidonate by phospholipids and TG in brain subcellular membranes is affected due to sample storage, exposure to elevated temperature and the effects of BSA and saponin. This type of variance has been well-discussed by Baker and Chang (20) as additional factors, such as the use of pH buffers and exogenous fatty acids, may also affect the acyl-transfer activity. However, because incubation of subcellular membranes in this study was carried out in a well-defined condition using the same buffer, these factors would not have affected the comparison of acylation profiles exhibited by these membranes. The effect of saponin on enhancing the acyltransfer to phospholipids and inhibiting the transfer to TG, again indicates that these two types of acyltransferases have different properties. The fact that both synaptosomes and plasma membranes respond similarly to saponin further suggests that along with its membrane permeability properties, this compound also alters the membrane microenvironment resulting in a change in acyltransferase activity.

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REFERENCES

1. Lands, W.E.M., and Crawford, C.G. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A., ed.) Vol. 2, pp. 3–85, Plenum Press, New York, NY.
2. Sun, G.Y., Su, K.L., Der, O.M., and Tang, W. (1979) *Lipids 14,* 229–235.
3. Corbin, D.R., and Sun, G.Y. (1978) *J. Neurochem. 30,* 77–82.
4. Deka, N., Sun, G.Y., and MacQuarrie, R. (1986) *Arch. Biochem. Biophys. 246,* 554–563.
5. Tang, W., and Sun, G.Y. (1985) *Neurochem. Res. 10*(10), 1343–1353.
6. Baker, R.R., and Chang, H-Y. (1983a) *Biochim. Biophys. Acta 752,* 1–9.
7. Baker, R.R., and Chang, H-Y. (1983b) *Can. J. Biochem. Cell Biol. 61,* 1265–1271.
8. Cook, H.W., Clarke, J.J.R., and Spence, M.W. (1982) *J. Lipid Res. 23,* 1292–1300.
9. Cook, H.W., and Spence, M.W. (1985) *Can. J. Biochem. Cell Biol. 63,* 919–926.
10. Strosznajder, J., Foudin, L., Tang, W., and Sun, G.Y. (1983) *J. Neurochem. 40,* 84–90.
11. Sun, G.Y., Corbin, D.R., Wise, R.W., and MacQuarrie, R. (1979) *Int. J. Biochem. 10,* 557–563.
12. Sun, G.Y., Huang, H-M., Kellecher, J.A., Stubbs, E.B. Jr., and Sun, A.Y. (1988) *Neurochem. Int. 12*(1), 69–77.
13. Booth, R.F.G., and Clark, J.B. (1981) *Biochem. J. 176,* 365–370.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265–275.
15. Sun, G.Y. (1988) in *Neuromethods* (Boulton, A., Baker, G., and Horrocks, L., eds.) Vol. 7, The Humana Press, Inc., Clifton, NJ (in press).
16. Horrocks, L.A., and Sun, G.Y. (1972) in *Research Methods in Neurochemistry* (Marks, N., and Rodnight, R., eds.) Vol. 1, pp. 223–231, Plenum Publishing Corporation, New York, NY.
17. Sun, G.Y. (1970) *J. Neurochem. 17,* 445–446.
18. Su, K.L., and Sun, G.Y. (1978) *J. Neurochem. 51,* 1043–1047.
19. Chakravarthy, B.R., Spence, M.W., and Cook, H.W. (1986) *Biocheim. Biophys. Acta 879,* 264–277.
20. Baker, R.R., and Chang, H-Y. (1986) *Biochem. Cell Biol. 64,* 1–7.

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Eicosanoid Synthesis in 7,12-Dimethylbenz(a)anthracene-induced Mammary Carcinomas in Sprague-Dawley Rats Fed Primrose Oil, Menhaden Oil or Corn Oil Diet

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The comparative effects of high-fat diets (20%, w/w) on eicosanoid synthesis during mammary tumor promotion in 7,12-dimethylbenz(a)anthracene (DMBA)-induced rats were studied using diets containing 20% primrose oil (PO), 20% menhaden oil (MO) or 20% corn oil (CO). Sprague-Dawley rats fed the PO or MO diet had 21% or 24% fewer adenocarcinomas, respectively, than rats fed the CO diet. Histologically (i.e., mitotic figures, inflammatory cell infiltration and necrosis), the CO-fed rats exhibited the highest frequency of changes within tumors. Plasma fatty acid composition was significantly altered by diet, reflecting the composition of the oils which were being fed. Only the plasma of PO-fed rats contained detectable levels of gamma-linolenic acid (GLA). Arachidonic acid (AA) levels were significantly higher ($p < 0.05$) in PO-fed than in CO- or MO-fed rats. MO-fed rats had significantly higher levels of plasma palmitic acid, while palmitoleic, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids were detected only in MO-fed rats. As expected, linoleic acid (LA) and AA levels were lower ($p < 0.05$) in the MO-fed rats than in PO- or CO-fed groups. The plasma of the CO-fed rats contained significantly higher levels of oleic acid. Eicosanoid synthesis in mammary carcinomas of rats fed the 20%-fat diets was 2–10 times higher than in mammary fat pads of control rats. The synthesis of PGE_1 and LTB_4 was significantly ($p < 0.05$) higher in PO-fed rats than in CO-fed or MO-fed rats, although PGE values were significantly ($p < 0.05$) higher in CO-fed rats than in MO or PO groups. The synthesis of eicosanoids in both mammary fat pads and mammary carcinomas of MO-fed rats was lower ($p < 0.05$) than in tissues of rats fed either CO or PO diets due to less AA precursor being fed and/or to competition between n-6 and n-3 fatty acids for cyclooxygenase and lipoxygenase. The ratios of monoenoic to dienoic eicosanoids in both mammary fat pads and mammary carcinomas were higher in the PO group than in the MO or CO groups. These results suggest that inclusion of GLA (PO feeding) or EPA and DHA (MO feeding) in the diet may decrease malignancy by altering eicosanoid profiles.

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The development of carcinogen-induced, spontaneous and transplantable mammary tumors is enhanced by the ingestion of diets containing elevated levels of polyunsaturated fats, especially *cis*-linoleic acid (1–4). Laboratory evidence suggests that the major influence of diets rich in polyunsaturated fat occurs during the promotional phases of chemically induced carcinogenesis. However, the exact mode of action for these effects has not been established. It has been suggested that the effect of dietary fat could be mediated by a change in the host defense system. One mechanism by which this could occur is via an increased synthesis of dienoic eicosanoids, i.e., PGE_2 (5). Vegetable oils such as corn oil (CO) or primrose oil (PO) contain high levels of linoleic acid (LA) (18:2n-6), the precursor of arachidonic acid (AA) (20:4n-6) and ultimately of the dienoic eicosanoids (prostaglandins and thromboxane) and tetraene leukotrienes. Moreover, some inhibitors of prostaglandin synthesis have been shown to prevent the growth-promoting effect of dietary polyunsaturated fatty acids on 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumorigenesis in rats (6).

Eicosapentaenoic acid (EPA) (20:5n-3), found in most fish oils and marine lipids, is the precursor of trienoic eicosanoids (prostaglandins and thromboxane) and pentaene leukotrienes. The EPA structure is similar to arachidonic acid (AA), except for one additional double bond at the 3-position relative to the terminal methyl of the chain. EPA has been shown to competitively inhibit AA metabolism by cyclooxygenase and lipoxygenase and give rise to its own products, some of which have attenuated activity (7).

A number of recent reports (8–11) have indicated that 20% or 10% menhaden oil (MO)-supplemented diets containing 0.34% or 0.09% LA, respectively, reduced tumor incidence and growth in the pancreas and mammary glands. Braden and Carroll (12) showed decreased DMBA-mammary tumor incidence and an increased tumor latency in rats fed 20% and 10% MO diets containing 0.4% and 0.2% LA, respectively, compared with rats fed either a 20% (11.2% LA) or a 10% (5.6% LA) corn oil diet. It has been suggested (13,14) that diets containing high levels of n-3 fatty acids should contain additional essential fatty acid (EFA) because the n-3 and n-6 fatty acids compete for the same enzymes, therefore requiring a higher EFA intake.

Primrose oil, in addition to 75% LA, contains 9% gamma-linolenic acid (GLA) (18:3n-6) that is rapidly converted to dihomogamma-linolenic acid (DGLA) (20:3n-6), the precursor of monoenoic eicosanoids. Data from our laboratory (15) and from others (16,17) have shown that a PO diet inhibits the growth of mammary adenocarcinomas in rats. Changing the ratio of DGLA/AA to shift eicosanoid synthesis toward monoenoic eicosanoids has been suggested as a mechanism for that inhibition (15).

This study was conducted to determine the effect of diets containing 20% PO, 20% MO or 20% CO on

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Abbreviations: AA, arachidonic acid; CO, corn oil; DGLA, dihomogamma-linolenic acid; DHA, docosahexaenoic acid; DMBA, 7,12-dimethylbenz(a)anthracene; D6D, delta-6 desaturase; EFA, essential fatty acid; EPA, eicosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid; MO, menhaden oil; PO, primrose oil; RIA, radioimmunoassay.

eicosanoid synthesis during tumor promotion in DMBA-induced mammary carcinomas, as well as in mammary fat pads of control rats.

MATERIAL AND METHODS

Diets, feeding and tumor induction. Female Sprague-Dawley rats, 40 days old, were purchased from Harlan (Madison, WI). All animals were housed in suspended metal cages in a temperature-regulated (23 ± 0.5°C) and light-controlled (12 hr light and 12 hr dark) room and were fed standard rat chow (Ralston Purina Co., St. Louis, MO). At 50 days of age, each rat was given a single intragastric 10 mg dose of DMBA (Sigma Chemical Company, St. Louis, MO) in 0.5 ml corn oil. Sham-treated rats received 0.5 ml corn oil. After DMBA administration, the animals were placed in 10 × 16-inch plastic cages on Absorb Dri litter and housed two per cage for the duration of the experiment. At 21 days post-DMBA administration, the rats were randomly divided into three groups of 26 rats each and fed diets containing either 20% CO, PO or MO. Sham-control rats were divided into three groups of 10 rats each and fed the same diets.

Ten- or 20-kg batches of the 20%-fat diets were prepared by ICN Nutritional Biochemical (Cleveland, OH), cold pressed into jumbo pellets and sealed under nitrogen. The diets were shipped second-day delivery by United Parcel Service at two-week intervals. Upon arrival, 1-kg bags of pellets were placed in Seal-N-Save bags, flushed with nitrogen, sealed and stored frozen at −20°C until used. Measured amounts of frozen diet were placed in the rat-cage food dispenser each morning. Uneaten pellets were discarded. The formulation of the diet was based on the AIN 76 semipurified rat diet and has been reported previously (15). Alpha-tocopherol was added with the AIN vitamin mix so that each diet contained 110 IU of vitamin E/kg of diet. All diets were isocaloric and contained the recommended level of nutrients with a constant amount per kilocalorie of casein, fat, carbohydrate, salts, vitamins and fiber. The fatty acid composition of the oils in the diets is given in Table 1. The 20%-CO or PO diet contained 12% (LA) or 16.8% (LA + GLA) EFA, respectively. The 20%-MO diet contained 0.8% EFA (1.81% LA plus 2.3% AA). The National Research Council (18) recommends, for maximum growth in the male rat, the level of EFA should be 0.6% of the diet and, in the female rat, the level of EFA should be 0.22% of the diet. However, when the diet contains high levels of n-3 fatty acids, the EFA requirement may be higher due to competition of n-6 and n-3 fatty acids for the same enzymes (13,14). The primrose oil was provided by Dr. David Horrobin of the Enfamol Research Institute (Kentville, Nova Scotia) and the menhaden oil by Dr. Tony Bimbo (Zapata Haynie Corp., Reedville, VA). The corn oil was purchased from Seaway Foods, Inc. (Cleveland, OH).

The rats were weighed weekly and palpated for the presence of tumors. The size and location of each tumor was noted. One rat fed CO and one rat fed MO were found dead before termination of the experiment. At 16 weeks post-DMBA, all surviving rats were killed using CO_2 gas and weighed, had blood drawn from the heart and then, the weight of the livers was recorded. The tumors were counted, location was noted and then, they were excised and weighed. Sections of each tumor were quickly frozen on dry ice and stored at −80°C for eicosanoid analysis. Two sections from each tumor were placed in 10% buffered formalin for histological examination. The blood was centrifuged at 2,000 rpm and the plasma stored briefly at 4°C.

TABLE 1

Fatty Acid Composition of Oils (%)

Fatty acid	Corn oil[a]	Primrose oil[b]	Menhaden oil[c]
14:0	—	—	8.35
16:0	11.2	6.5	15.17
16:1	—	0.2	11.62
16:2	—	—	2.37
16:3	—	—	1.96
16:4	—	—	1.73
18:0	2.1	1.5	2.67
18:1	25.0	7.5	9.5
18:2n-6	59.9	75.0	1.81
18:3n-6	0.5	9.0	—
18:3n-3	—	—	1.82
18:4	—	—	3.47
20:1	—	—	1.32
20:4n-6	—	—	2.3
20:5n-3	—	—	16.03
22:5	—	—	3.92
22:6n-3	—	—	10.83
Others	0.1	—	4.37

[a]The corn oil analysis was provided by ICN Nutritional Biochemical, Cleveland, OH.

[b]The primrose oil analysis was provided by Efamol Research Corp., Kentville, Nova Scotia, Canada.

[c]The menhaden oil analysis was provided by Zapata Haynie Corp., Reedville, VA.

Lipid profiles. Total plasma lipids were extracted in chloroform/methanol (2:1), after the method of Folch et al. (19). Methyl esters of fatty acids were prepared with boron trifluoride, as described by Morrison and Smith (20). These procedures have been described in detail by Abou-El-Ela et al. (15).

The fatty acid methyl esters were analyzed using a Hewlett-Packard Model 402 gas chromatograph equipped with a flame ionization detector and connected to a Spectra-Physics Model 4270 recording integrator. Chromatography was performed on a 6 ft × 2 mm glass column containing 10% SP-2330 on 100/120 chromosorb W AW (Supelco Inc., Bellefonte, PA).

Eicosanoid analyses. Malignant mammary tumors from DMBA-treated rats and fat pads of control animals were finely minced and an appropriate tissue aliquot was incubated in 1 ml ice-cold Krebs buffer (containing 2.4 M NaCl, 0.1 M KCl, 0.01 M $MgSO_4$, 0.06 M $CaCl_2$, 0.02 M KH_2PO_4, 0.03 M $NaHCO_3$ and 0.01 M dextrose, pH 7.4) for 1 hr at 37°C under 95% O_2/5% CO_2. The reaction was stopped by acidification to pH 3 with 0.8 M phosphoric acid and the tissue-buffer mixture was extracted once with 4 volumes of ethyl acetate by vigorous shaking (21). The organic phase was removed, and 50 μl of 0.1 M Tris-HCl buffer (pH 7.4) was added. The organic phase was evaporated under nitrogen, and the residue was stored, sealed under nitrogen at −80°C. The samples were appropriately diluted in assay buffer and the stable metabolites of prostacyclin; 6-keto-$PGF_{1\alpha}$ of prostaglandin E;

bicyclic PGE and LTB_4 were analyzed by radioimmunoassay (RIA) using kits purchased from Amersham Corp. (Arlington Heights, IL). The sensitivities of these kits are 12.5, 43 and 14 pg for LTB_4, PGE, and 6-keto-$F_{1\alpha}$, respectively. The PGE_2 antibody from Amersham showed 100% cross-reactivity with PGE_1; thus, PGE_2 is designated as PGE, indicating that both PGE_2 and PGE_1 were assayed in the tissue extracts when the Amersham PGE_2 kit was used. RIA kits for PGE_1 were purchased from Chemical Assays Labs (Cambridge, MA). The lowest detectable level of PGE_1 is 8 pg. PGE_1 antibody cross-reactivity with PGE_2 was 31%. Eicosanoid values are expressed as ng of eicosanoid synthesized per gram of tissue per hour.

Histopathology. A complete necropsy examination was performed on each rat and gross lesions were evaluated. Mammary tumors were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 6 microns and stained with hematoxylin and eosin. Each tumor was described histologically and a diagnosis of each tumor was ascertained, according to the criteria of van Zwieten (22). Histologic descriptions included notations of necrosis, inflammation and anaplasia, and when possible, the invasive or noninvasive nature of the tumor was given.

Statistical analysis. Statistical differences in tissue eicosanoid levels, plasma lipids, body and liver weights and tumor latency were determined by one-way analysis of variance at 0.05 level of significance. Among groups, comparison of tumor burden per tumor-bearing rat involved one-way analysis of variance following square root transformation.

RESULTS

Effect of 20% PO, CO and MO diets on mammary tumorigenesis. Although the number of rats with tumors was similar in the animals fed the three different diets (Table 2), the histopathological data indicate that there was an effect of fat type on the malignancy of those tumors (Table 3). Rats receiving the 20%-PO diet had 21% fewer malignant tumors (64 tumors in 26 rats compared with 78 tumors in 25 rats for CO), and rats fed 20%-MO had 24% fewer malignant tumors (59 tumors in 25 rats compared with 78 for CO) than rats fed the 20%-CO diet. The latent period for tumor development (calculated by averaging the week of tumor appearance for all palpable tumors within a group) was significantly longer ($p < 0.05$) and tumor burden per tumor-bearing rat was significantly ($p < 0.05$) lower in rats fed the PO diet compared with CO-fed rats (Table 2). In the other histopathological parameters measured (mitotic figures, inflammatory cell infiltration and necrosis), the 20%-CO-fed rats exhibited the highest frequency of these changes within tumors (Table 3). The classification of tumors revealed similar diagnoses in all groups.

The animals were thoroughly examined at necropsy; there were no gross signs of EFA deficiency, i.e., hair loss, scaly skin, necrosis of the tail or low body weight.

TABLE 2

The Effect of Feeding 20%-CO, PO or MO Diets On Mammary Tumorigenesis Induced by DMBA

Diet	No. of surviving rats	No. of rats with tumors	Total no. of tumors[a]	Total no. of malignant tumors	Latent period[b] (wks)	Tumor burden/ tumor bearing rat[b] (g)
20% PO	26	21	68	64	10.6 ± 0.5[c]	2.0 ± 0.33[c]
20% CO	25	22	83	78	9.3 ± 0.3[d]	3.2 ± 0.40[d]
20% MO	25	21	64	59	10.1 ± 2.3[c,d]	2.3 ± 0.42[c,d]

[a]These values include both malignant and benign tumors.

[b]Values are mean ± SEM.

[c,d]Means which are significantly different ($p < 0.05$) are followed by a different superscript.

TABLE 3

Histopathological Classification of DMBA-induced Mammary Tumors in Rats Fed 20%-CO, PO, or MO Diets and Killed at 16 Weeks Post-DMBA

	Diagnosis of tumors[a] (number of tumors)					Number of tumors with mitotic figures (avg/hpf)		Tumors with stromal inflammatory cell infiltration	Number of tumors with necrosis
Diet	TPC	CTC	TA	FA	AL	<3	3 to 5+		
20% PO (26[b])	59	5	3	1	0	61	7	62	6
20% CO (25)	75	3	4	1	0	67	16	80	8
20% MO (25)	57	2	3	1	1	57	7	59	2

[a]Malignant tumors = Tubulopapillary carcinoma (TPC) and compact tubular carcinoma (CTC). Benign tumors = Tubular adenoma (TA), fibroadenoma (FA) and adenolipoma (AL).

[b]No. in ( ) = no. of rats/diet group.

Although the EFA content of the PO diet was higher than that of the CO diet, the body weights (Table 4) of PO-fed rats were significantly ($p < 0.05$) lower than those fed CO or MO diets. The liver weights of the MO-fed rats were significantly higher than those of the CO- or PO-fed rats. Therefore, liver to body weight ratios for the MO-fed rats were significantly higher than those for CO-fed rats (TAble 4). In a separate study on these animals (23), we reported that feeding a 20%-MO diet induces liver mixed function oxidases and glutathione-S-transferase.

Effect of 20% PO, CO or MO on eicosanoid synthesis in mammary fat pads of control rats and mammary tumors of DMBA-treated rats. In sham-treated animals, synthesis of PGE_1 and LTB_4 (ng/g wet wt/hr) in mammary fat pads was significantly ($p < 0.05$) higher in PO-fed rats (Table 5) than in MO- or CO-fed rats. Although PGE was significantly ($p < 0.05$) higher in CO-fed animals than in MO or CO groups, eicosanoid synthesis (PGE, 6-keto-$PGF_{1\alpha}$ and LTB_4) was significantly lower ($p < 0.05$) in MO-fed rats. Even though eicosanoid profiles in the mammary fat pads followed the same trends as the profiles in the malignant mammary tumors, rates of synthesis of all eicosanoids were 2–10 times higher in mammary tumors than in mammary fat pads of control animals (Table 5). However, the ratios of PGE_1/PGE, PGE_1/6-keto-$PGF_{1\alpha}$, as well as PGE_1/LTB_4, which were elevated in rats fed the PO diet, were similar in both control and DMBA-treated animals. In mammary tumors, PGE and 6-keto-$F_{1\alpha}$ synthesis rates were significantly ($p < 0.05$) higher in tumors of CO-fed rats than in tumors of PO- or MO-fed rats, and PGE_1 values were significantly ($p < 0.05$) higher in PO-fed rats. PGE_1, PGE, 6-keto-$PGF_{1\alpha}$ and LTB_4 synthesis rates were lower in MO-fed rats than in CO or PO groups.

Effect of dietary fat on plasma fatty acid profile of control and tumor-bearing rats. The plasma fatty acid profile of control and tumor-bearing rats (Table 6) indicated that the quantitative and qualitative differences in the dietary fatty acid content of PO, CO and MO produced relatively selective alterations in the fatty acid content of the plasma that reflected the diet being fed. The plasma levels of palmitic acid (16:0) were elevated significantly ($p < 0.05$) in rats fed the MO diet and reduced in rats fed PO, relative to levels in rats fed CO for both DMBA-treated and control animals. Palmitoleic acid (16:1) was detected only in the plasma of MO-fed animals, and stearic acid (18:0) was high in the plasma of PO-fed control animals. Oleic acid (18:1) was significantly ($p < 0.05$) higher in the CO-fed, lowest in PO-fed and intermediate in MO-fed animals. As expected, plasma LA concentrations from rats fed PO and CO diets were significantly ($p < 0.05$) higher than that of rats fed the MO diet. GLA and AA levels were significantly ($p < 0.05$) higher in rats fed PO compared with those fed CO or MO. On the other hand, EPA and DHA (22:6n-3) levels were detectable only in MO-fed rats.

TABLE 4

The Effect of Diet on Body and Liver Weights in Tumor-Bearing Rats

Diet	Final body weight[a] (g)	Liver weight[a] (g)	Liver/body weight Ratio (%)
PO	258.8 ± 2.81[b]	10.58 ± 0.28[b]	4.1 ± 0.09[b,c]
CO	275.5 ± 5.73[c]	10.68 ± 0.40[b]	3.9 ± 0.14[b]
MO	281.8 ± 3.6[c]	12.8 ± 0.48[c]	4.5 ± 0.2[c]

[a]Values are mean ± SEM.

[b,c]Means which are significantly different are followed by a different superscript ($p < 0.05$).

DISCUSSION

Although tumor incidence was similar in rats fed the MO, PO and CO diets, the number of malignant tumors was reduced by 24% and 21% in the MO- and PO-fed rats, respectively, compared with malignant tumors recovered from rats fed the 20%-CO diet. Moreover, the frequency of mitosis and the extent of necrosis and inflammation within tumors were higher in CO-fed rats compared with

TABLE 5

The Effect of 20% Dietary Fat on Eicosanoid Synthesis in Mammary Fat Pads of Control and Tumors of DMBA-Treated Rats

Diet	PGE_1[a]	PGE[a]	PGE_1/PGE	6-keto-$PGF_{1\alpha}$[a]	PGE_1/6-keto-$PGF_{1\alpha}$	LTB_4[a]	PGE_1/LTB_4
20% PO (Control)	63.7 ± 6.9[b]	56.0 ± 3.7[b]	1.14	236.7 ± 46.8[b]	0.27	66.7 ± 3.5[b]	0.96
20% CO (Control)	27.0 ± 7.0[c]	154.0 ± 15.1[c]	0.18	214.0 ± 9.7[b]	0.13	54.3 ± 5.5[c]	0.50
20% MO (Control)	8.4 ± 3.0[c]	121.3 ± 3.3[d]	0.07	51.7 ± 3.2[c]	0.16	40.7 ± 0.7[d]	0.21
20% PO (Tumor bearing)	388.9 ± 22.8[b]	272.4 ± 31.9[b]	1.4	1038.4 ± 142.4[b]	0.37	116.6 ± 8.9[b]	3.3
20% CO (Tumor bearing)	149.5 ± 20.3[c]	727.9 ± 157.1[c]	0.2	1740.3 ± 228.9[c]	0.09	103.6 ± 12.1[b]	1.4
20% MO (Tumor bearing)	106.9 ± 17.5[c]	234.2 ± 55.4[b]	0.5	437.6 ± 54.2[d]	0.24	61.5 ± 7.1[c]	1.7

[a]Values are expressed as mean in ng/g tissues/hr ± SEM (n = 6 for fat pads, and n = 13–16 for tumors).

[b,c,d]Means which are significantly ($p < 0.05$) different are followed by a different superscript. Control values were compared with other controls, and values from tumor-bearing rats were compared with values from other tumor-bearing rats.

TABLE 6

The Effect of 20% Dietary Fat on Plasma Fatty Acid Profiles of Tumor-bearing and Control Animals[a]

Diet	Percentage composition									
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5n-3	C22:6n-3
POC (Control)	2.19 ± 1.0[b]	11.6 ± 1.0[b]	ND	16.3 ± 1.0[b]	4.7 ± 0.5[b]	30.1 ± 1.4[b]	2.4 ± .3	32.7 ± 1.0[b]	ND	ND
COC (Control)	.15 ± 0.1[b]	15.2 ± 0.6[c]	ND	10.4 ± 0.7[c]	16.3 ± 0.9[c]	34.9 ± 2.9[b]	ND	26.0 ± 1.6[c]	ND	ND
MOC (Control)	1.74 ± 0.3[b]	23.4 ± 0.7[d]	8.7 ± 0.7	11.2 ± 0.5[c]	12.2 ± 0.5[d]	3.3 ± 1.5[c]	ND	10.2 ± 0.5[d]	24.8 ± 1.0	7.3 ± 0.4
PO (Tumor-bearing)	1.4 ± 0.7[b]	12.2 ± 0.6[b]	ND	12.3 ± 1.3[b]	5.2 ± 0.4[b]	31.9 ± 2.1[b]	2.3 ± 0.7	33.4 ± 1.8[b]	ND	ND
CO (Tumor-bearing)	.76 ± 0.3[b]	15.4 ± 0.4[c]	ND	11.3 ± 0.7[b]	14.2 ± 0.6[c]	32.4 ± 1.1[b]	ND	25.7 ± 1.5[c]	ND	ND
MO (Tumor-bearing)	1.3 ± 0.3[b]	25.1 ± 0.5[d]	4.1 ± .7	14.0 ± 0.5[b]	11.3 ± 0.5[d]	3.0 ± 0.2[c]	ND	13.1 ± 0.7[d]	27.1 ± 1.5	7.0 ± 0.4

[a] Values are expressed as mean ± SEM (n = 10).

[b,c,d] Means which are significantly different are followed by a different superscript ($p < 0.05$). Control values were compared with other controls, and values from tumor-bearing rats were compared with values from other tumor-bearing rats.

ND, Not detected.

MO- or PO-fed rats. These changes denote a more rapid tumor growth and a greater, although altered, immunologic reactivity than was present in tumors from PO- or MO-fed rats. Feeding a 20%-PO diet significantly extended tumor latency and reduced tumor burden compared with feeding the 20%-CO diet. Although tumor incidence was unchanged with any of the three diets in this study, in a previous study in our laboratory (15), rats fed a 20%-PO diet had significantly decreased incidence of DMBA-induced mammary tumors compared with those fed a 20%-CO diet. Ip et al. (2) showed that mammary tumorigenesis was very sensitive to linoleate intake and increased proportionately in the range of 0.5% to 4.4% of dietary linoletate. The 20%-MO diet contained 0.8% EFA, and the 20%-CO or PO diets contained 12% or 16.8% EFA, respectively. The 20%-PO diet contained 4.8% more EFA than the CO diet, yet rats fed PO diet had 21% fewer malignant tumors, longer latency and reduced tumor burden. On the other hand, the 20%-MO diet with 0.8% EFA had 24% fewer malignant tumors than CO-fed rats. Karmali (24) showed that MO provides greater inhibition of tumorigenesis, when up to 8% CO is present in the diet. In a recent study in our laboratory (unpublished data), when a combination of 15% MO and 5% CO (3.62% EFA) was fed to the rats, mammary tumor incidence was reduced by 27% and the number of malignant tumors was reduced by 37% compared with results from rats fed a 20%-CO diet. It is important to note that the n-3/n-6 fatty acid ratio for 15%-MO + 5%-CO diet was 1.2, but the n-3/n-6 ratio in the 20%-MO diet was 7.0. Thus, it appears from our data and others (24) that optimal dietary ratios of n-3/n-6 fatty acids must be maintained to obtain significant inhibition of tumorigenesis by omega-3 fatty acids. Moreover, it seems possible that tumor promotion and malignancy can be reduced by manipulation of both the types and levels of dietary polyunsaturated fatty acids, even in the presence of relatively high linoleate levels.

Dietary fat can play a major role in eicosanoid production and in modulation of the immune system (25). The n-3 fatty acids of MO appear to inhibit both cyclooxygenase and lipoxygenase (7), although PO enhances monoenoic eicosanoid synthesis by bypassing delta-6 desaturase (D6D), the rate-limiting enzyme for the synthesis of DGLA to 1-series PG (Fig. 1). By feeding three kinds of high-fat diet, we have been able to manipulate eicosanoid production that appears to alter tumorigenesis. Feeding a diet containing 20% CO results in high levels of dienoic eicosanoids, but feeding a 20%-MO diet depresses eicosanoid synthesis either by inhibiting both cyclooxygenase and lipoxygenase or because less AA precursor is being fed in the diet. The feeding of PO depressed dienoic eicosanoid synthesis and enhanced monoenoic eicosanoids and leukotriene synthesis compared with CO feeding. Furthermore, the ratios of monoenoic to dienoic eicosanoids or 4-series leukotrienes (PGE_1/PGE, PGE_1/6-keto-$PGE_{1\alpha}$, PGE_1/LTB_4) were increased by feeding PO. This may account for the reduction in malignancy that seemed to accompany the feeding of PO. Honn et al. (26,27) have hypothesized that selective manipulation of the AA cascade could be used to control tumor promotion and metastasis. Furthermore, they postulated that eicosanoid ratios, rather than absolute values of eicosanoids, determine cellular events in promotion and metastasis.

PGE_2 has been shown to induce suppressor T-cell proliferation, activation of natural killer cells and production of lymphokines (28). When released extracellularly, products of both lipoxygenase and cyclooxygenase pathways are postulated to contribute to development of the inflammatory response (28). Although LTB_4 and PGE_2 may act synergistically to increase vascular permeability, LTB_4 and prostaglandins may exert opposing effects on neutrophil function. In stimulated neutrophils, PGE_2, PGE_1 and 6-keto-$PGF_{1\alpha}$ effectively inhibit adherence, aggregation, calcium mobilization, superoxide generation and lysosomal enzyme release (25). Because

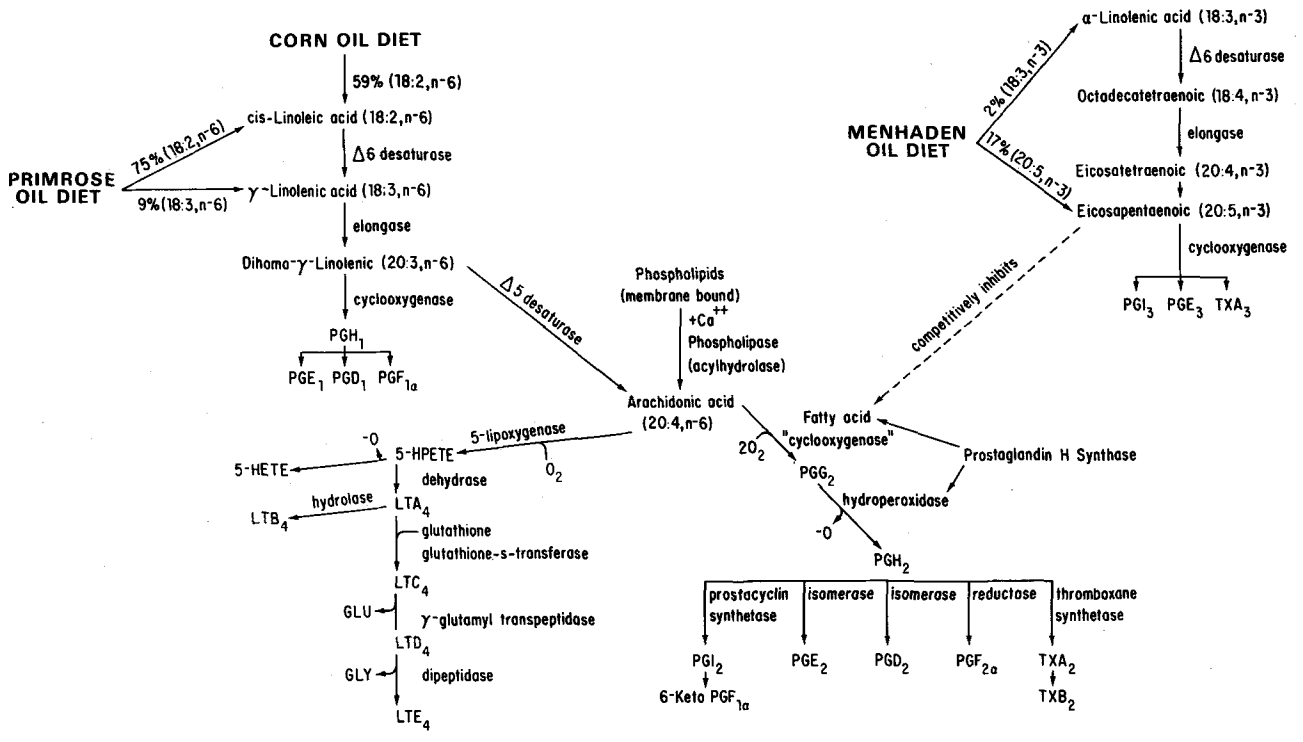

FIG. 1. Scheme for the metabolism of corn oil, primrose oil and menhaden oil to eicosanoids.

LTB_4 increases neutrophil adherence, Ham et al. (29) speculated that production of prostaglandins by endothelial cells will suppress adhesion of neutrophils to the vascular endothelium by inhibiting LTB_4 production, Korchak and Abramson (25) have observed that LTB_4 activation of neutrophils was significantly depressed in the presence of PGE_1. Thus, the reduction in number of malignant tumors observed in PO-fed rats may be mediated by increased synthesis of PGE_1 that may, in turn, alter immune responses by opposing LTB_4. The finding that a MO diet favors the immunologic generation of LTB_5, which has attenuated biological activity compared with LTB_4, suggests that EPA-enriched tissues may exhibit less proinflammatory activity than EPA-poor tissues (30). Feeding 20% MO in this study decreased all dienoic eicosanoid levels, as well as the proinflammatory leukotriene, LTB_4. In order to explain the mechanism of inhibition of tumorigenesis by n-3 fatty acids, optimal trienoic/dienoic eicosanoid ratios should be established. The lack of adequate methodology to measure trienoic eicosanoids is currently the limitation.

In summary, the present study shows that eicosanoid synthesis can be selectively modified by feeding n-3 or n-6 fatty acid precursors. Furthermore, inclusion of GLA or substitution of EPA and DHA for LA in the diet appears to decrease the malignancy of mammary tumors induced by DMBA. Our data suggest that the reduction in malignancy by dietary PO may be accomplished by decreasing the "protumor" dienoic eicosanoids and/or increasing monoenoic eicosanoids in tissues, but the effect of dietary MO may be mediated through depression of the production of dienoic eicosanoids and 4-series leukotrienes. The mammary tumor-promoting effect of dietary fat, specifically CO, may be mediated by overproduction of selective dienoic eicosanoids that modulate the immune response. Further studies are needed to establish the subtle mechanism(s) of fat types and levels in mammary cancer. In particular, the question of the optimal dietary ratios of n-3 to n-6 fatty acids needs to be addressed.

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REFERENCES

1. Welsch, C. (1985) *Cancer Res. 45,* 3415-3443.
2. Ip, C., Carter, C.A., and Ip, M.M. (1985) *Cancer Res. 45,* 1997-2001.
3. Lee, S.Y., and Rogers, A.E. (1985) *Nutr. Res. 3,* 361-371.
4. Rao, G.A., and Abraham, S. (1976) *J. Natl. Cancer Inst. 56,* 431-432.
5. Karmali, R.A. (1986) in *Dietary Fat and Cancer* (Ip, C., Birt, D.F., Rogers, A.E., and Mettlin, C., eds.) pp. 687-697, Alan R. Liss Inc., New York, NY.
6. Carter, C.A., Milholland, R.J., Shea, W., and Ip, M.M. (1983) *Cancer Res. 43,* 3559-3562.
7. Karmali, R.A. (1987) *Am. J. Clin. Nutr. 45,* 225-229.
8. O'Connor, T.P., Roebuck, B.D., Peterson, F., and Campbell, T.C. (1985) *J. Natl. Cancer Inst. 75,* 959-962.
9. Jurkowski, J.J., and Cave, Jr., W.T. (1985) *J. Natl. Cancer Inst. 74,* 1145-1150.
10. Gabor, H., and Abraham, S. (1986) *J. Natl. Cancer Inst. 76,* 1223-1229.
11. Cave, Jr., W.T. and Jurkowski, J.J. (1987) in *Proceedings of the*

AOCS Short Course on Polyunsaturated Fatty Acids and Eicosanoids (Lands, W.E.M., ed.) pp. 261-266, American Oil Chemists' Society, Champaign, IL.
12. Braden, L.M., and Carroll, K.K. (1986) *Lipids 21,* 285-288.
13. Crawford, M.A. (1987) in *Proceedings of the AOCS Short Course on Polyunsaturated Fatty Acids and Eicosanoids* (Lands, W.E.M., ed.) pp. 270-294, American Oil Chemists' Society, Champaign, IL.
14. Holman, R.T. (1986) *Prog. Lipid Res. 25,* 29-39.
15. Abou-El-Ela, S.H., Prasse, K.W., Carroll, R., and Bunce, O.R. (1987) *Lipids 22,* 1041-1044.
16. Ghayur, T., and Horrobin, D.F. (1981) *IRCS Med. Sci. 9,* 582.
17. Karmali, R.A., Marsh, J., and Fuchs, C. (1985) *J. Nutr. Growth Cancer 2,* 41-51.
18. National Research Council (1978) in *Nutrient Requirements of Laboratory Animals* (3rd edn.) National Academy of Sciences, Washington, DC.
19. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
20. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600-608.
21. Rydzik, R.M. (1984) in "Characterization and Pharmacological Properties of Unknown Eicosanoids," pp. 68, Ph.D. Dissertation, University of Georgia, Athens, GA.
22. Van Zwieten, M.J. (1984) in *The Rat as Animal Model in Breast Cancer Research* (Van Zwieten, M.J., ed.) pp. 53-134, Martinus Nijhoff, Boston, MA.
23. Wade, A.E., Dharwadkar, S., Bunce, O.R. and Abou El-Ela, S.H. (1988) *Drug Nutrient Interactions,* in press.
24. Karmali, R.A. (1987) in *Proceedings of the AOCS Short Course on Polyunsaturated Fatty Acids and Eicosanoids* (Lands, W.E.M., ed.) pp. 222-232, American Oil Chemists' Society, Champaign, IL.
25. Korchak, H.M., and Abramson, S.B. (1985) in *Prostaglandins and Immunity* (Goodwin, J.S., ed.) pp. 179-202, Martinus Nijhoff Publishing, Boston, MA.
26. Honn, K.V., Bockman, R.S., and Marnett, L.J. (1981) *Prostaglandins 21,* 833-864.
27. Honn, K.V., Busse, W.D., and Sloan, B.F. (1983) *Biochem. Pharmacol. 32,* 1-11.
28. Erickson, K.L. (1986) *J. Immunopharmacol. 8,* 529-543.
29. Ham, E.A., Soderman, D.D., Zanetti, M.E., Dougherty, H.W., McCauley, E., and Kuehl, F.A. (1983) *Proc. Natl. Acad. Sci. USA 80,* 4349-4353.
30. Lee, T.H., and Austen, K.F. (1986) *Adv. Immunol. 39,* 145-175.

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Esterifications of 1- and *rac*-2-Octanols With Selected Acids and Acid Derivatives Using Lipases

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Several aliphatic acids and their ethyl, isopropenyl and 2,2,2-trichloroethyl esters were allowed to react with 1- and 2-octanols catalyzed by commercial lipase preparations of porcine pancreas and the fungi *Candida rugosa*, *Aspergillus niger* and *Mucor miehei*.

Comparisons of reactivity of the acids and esters were made in common organic solvents using the primary alcohol. Reactions of octanoic acid and its esters with 2-octanol in hexane allowed an evaluation of stereoselectivity of the lipases with different substrates that carried the same (octanoyl) residue. A partial resolution of *rac*-2-hexadecanol with *A. niger* lipase is described, and the utility of lipase selectivities (stereo, positional, fatty acid and ester) is discussed with reference to the data presented.

Lipids 23, 955–960 (1988).

Fatty acid selectivities of commercial and purified microbial lipases continue to receive much attention (1,2). Such information would be useful for projected industrial reactions such as the synthesis of fatty acid esters and transformations of natural triglycerides (3–6). The effect of the alcohol or other residue attached to a given acyl substrate on lipase preferences for fatty acids has been studied less frequently, although the accumulated evidence (7–10) indicates that acylation of the enzyme may be promoted, and/or acylation may be made irreversible, by suitable choice of acylating agent. Moreover, the large number of lipase-mediated kinetic resolutions of alcohols (7) and the oft documented triglyceride position preferences of lipases (1) demonstrate the importance of the structure of the alcohol component in reactions that are catalyzed by these enzymes.

We have screened separately and compared several acid derivatives of acetic, octanoic and oleic acids in reactions with 1- and 2-octanol, in order to further assess the potential importance of the nonacyl portion of the substrate in lipase reactions. In the process, we found that a lipase of *Aspergillus niger* (Amano-Lipase K) also is useful for resolving secondary alcohols. In addition, we observed that the degree of stereobias exhibited by lipases can be affected sometimes by the nature of the group associated with the acid residue in the starting material. Lipase selectivities based on the data reported here are evaluated for utility in kinetic resolution of enantiomeric alcohols and in selective transesterification of primary alcohols.

MATERIALS AND METHODS

Gas liquid chromatography (GLC) was performed with a Shimadzu GC-Mini 2 instrument (Columbia, MD) using an SPB-1 column (0.25 mm i.d. × 30 m) fitted with a flame ionization detector and operated at column temperatures indicated herein, with a 50:1 split ratio and with He carrier gas. Chromatograms were recorded with either a Perkin-Elmer Model 023 Recorder (Norwalk, CT) or a Hewlett-Packard 3390 Recording Integrator (Avondale, PA). Infrared spectra (IR) were obtained on a Perkin-Elmer 1310 Spectrophotometer (3% solutions in CCl_4). Nuclear magnetic resonance spectra (NMR) were obtained in $CDCl_3$ using a JEOL JNM-GX 400 FT NMR spectrometer (Piscataway, NJ). Mass spectra (MS) were recorded with a Hewlett-Packard 5995 GC-MS System interfaced with an OV-1 column (0.25 mm i.d. × 12 m). Optical rotations were obtained using a Perkin-Elmer 141 Polarimeter.

Acetic acid was Baker Analyzed Reagent (Baker, Phillipsburg, NJ), all other organic acids were Aldrich Reagent Grade (Aldrich, Milwaukee, WI) and were employed without purification. Hydrocarbon standards for GLC were purchased and used as received, also. Ethyl and 2,2,2-trichloroethyl esters were synthesized in the usual manner from the alcohols and acids (Fisher esterification) or acid halides (triethylamine, CH_2Cl_2). They were distilled and characterized by IR, MS and GLC. Isopropenyl esters were prepared in slightly better yield, and more conveniently than previously reported (11), and an example is given as well. Solvents were purchased "distilled in glass," or were reagent grade and were distilled before use (acetone distilled from $KMnO_4$ and 1,2-dimethoxyethane distilled from lithium aluminum hydride). Diethyl ether was Analyzed Reagent-Anhydrous (Baker) and was used directly. The enzyme preparations by biological source (commercial source; company trade name; initial rate assay on olive oil emulsions given in μmol free fatty acid released, min^{-1}, mg^{-1}) were *C. rugosa* (Enzeco, none, 9.82) (Enzyme Development Corp.; New York, NY), *A. niger* (Amano; Lipase K; 11.2) (Amano, Troy, VA), *M. miehei* (NOVO; Lipase-3A; 5.33) (NOVO, Wilton, CT) and Porcine pancreatic lipase (Sigma; none; 15.6) (Sigma, St. Louis, MO).

Immobilization of lipases. Lipase-3A is on a resin (proprietary), as purchased. For ease in use, greater stability and potential for recovery, the other two fungal lipases were adsorbed onto Duolite, an ion-exchange resin available from Rohm and Haas Co. (Philadelphia, PA). Duolite (50 g) was washed sequentially with 2 × 100 ml of methanol, and then 2 × 100 ml of .05 N phosphate buffer at pH 7.0. The material was then swirled in 100 ml of the same buffer containing 5 g of the commercial lipase powder (30°C, 16 hr). The mixture was suction filtered and washed with 2 × 100 ml of reagent acetone. The granular product was dried under vacuum at 25°C.

Activities of the lipases in organic solvent before and after adsorption were evaluated as follows: 5 ml of wet hexane containing 0.50 mmol of 1-octanol and 0.25 mmol of octadecane, and 5 ml of wet hexane containing 0.50 mmol of octanoic acid were combined. Catalyst

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Abbreviations: ee, enantiomeric excess; GLC, gas liquid chromatography; IR, infrared spectra; MS, mass spectra or spectrometry; NMR, nuclear magnetic resonance spectra.

(20 mg of powder or 0.20 g of immobilized enzyme) was added, the reaction mixture was stirred at 30°C and samples were analyzed by GLC (200°C) at 1.0 and 2.0 hr. The measurements were duplicated and averaged. Lipase (powder activity in μmol of ester formed; hr^{-1}, mg; immobilized enzyme activity): *C. rugosa* (1.1, 0.13); *A. niger* (0.33, 0.35). The pancreatic lipase was used as a powder; a useful preparation on Duolite was not obtained. Lipase 3A is immobilized on an ion-exchange resin, as purchased.

Preparation of isopropenyl heptanoate. Heptanoic acid (50 ml), isopropenyl acetate (200 ml) and H_2SO_4 (0.3 ml) were heated under reflux for 5 hr. The mixture was cooled to ca. 25°C, stirred for 0.25 hr with 20 g of sodium acetate and 100 ml of anhydrous ether, and then filtered. The filtrate was concentrated on a rotary evaporator to remove ether and isopropenyl acetate. The concentrate was dissolved in 200 ml of ether and stirred for 2 hr with 25 g of $NaHCO_3$ and 50 ml of water. The organic phase was recovered and dried (Na_2SO_4). The solvent was removed, and the residue was distilled through a Vigreaux column collecting the fraction bp 87–93°C (20 mm): 19.2 g, 32.2% yield, IR 1750, 865 cm^{-1}, GC-MS *m/e* 113 (heptanoyl ion), no molecular ion obtained); ^{13}C NMR 14.09, 19.58, 22.60, 24.91, 29.01, 31.65, 34.37, 101.96, 153.01, 171.02. The product was contaminated with about 3–4% of the acetic heptanoic anhydride. Isopropenyl octanoate and oleate were similarly prepared; the latter was purified by column chromatography, as previously reported (11).

Esterifications of 1-octanol. Reaction mixtures were prepared that contained 0.50 mmol each of 1-octanol and the acylating reagent and 0.25 mmol of the hydrocarbon internal standard in 10 ml of wet solvent. Water-miscible solvents were fortified with 2 μl of distilled water. The enzyme formulation was added, and the reaction mixture was stirred at 30°C. Aliquots were analyzed by GLC—acyl residue (hydrocarbon standard, GLC temperature): acetyl (undecane, 120°C); octanoyl (octadecane, 200°C); oleoyl (tetracosane, 280°C). The amount of catalyst used was adjusted downward, as necessary, to reduce percentage conversion to less than 20% during the first hour and thereby provide a calculated velocity close to an initial rate assay (the rate that would be calculated from the initial linear portion of the rate curve, with an excess of substrate to assure saturation of enzyme active sites). Most reactions proceeded to less than 5% conversion under the conditions employed.

Esterifications of 2-octanol. Reaction mixtures were prepared containing 0.50 mmol each of *rac*-2-octanol and octanoic acid (ester) and 0.25 mmol of octadecane in 10 ml of wet hexane. The enzyme (0.20 g of formulated material or 225 mg of pancreatic lipase powder) was added, and the mixture was shaken at 30°C for 4–6 days. Analysis by GLC provided percentage conversion. The configurational analysis for residual 2-octanol was obtained, as previously described (12), using (S)-α-methylbenzylisocyanate (13) to produce chromatographically separable diastereomers to determine the enantiomeric excess (ee) of the unesterified alcohol.

Resolution of rac-*2-hexadecanol.* 2-Hexadecanol (2.94 g, 10 mmol) was shaken in a solution of wet hexane (50 ml) containing isopropenyl oleate (3.23 g, 10 mmol) and *A. niger* on Duolite (5 g) at 30°C for 14 days. The mixture was suction filtered, and the resin was washed with small portions of hexane. The combined organic phase was concentrated and chromatographed on silica gel, 60–200 mesh, 30 g (Grace Co., Baltimore, MD). The oleate ester was eluted with 2–5% ether-hexane, and the unreacted alcohol was eluted with 7.5–10% ether-hexane (1.36 g, 46.3% of 70.0 ee:S). The ester was saponified (20 ml of 50% 4 N KOH-methanol, reflux overnight) to produce alcohol (1.28 g, 43.5% of 86.6 ee:R). The ester, in other words, was 93.3% (R). The R-alcohol had α_D^{25}-4.0 (C = 4.7, EtOH). A midfraction was recovered with 5% ether-hexane that was mostly alcohol. The chromatographic conditions for the diastereomeric carbamates used to assign configurational purity to the alcohols were: column temperature 280°C, k's 4.167 (R,S-diastereomer), 4.375 (S,S-diastereomer) and a separation factor of 1.050.

RESULTS AND DISCUSSION

Esterification of 1-octanol. Acetic, octanoic and Z-9-octadecenoic (oleic) acids were allowed to react with 1-octanol in wet hexane at 30 ± 0.2°C with the following commercial lipase preparations: Enzeco *C. rugosa,* Amano Lipase K. *(A. niger),* NOVO Lipase 3A *(M. miehei)* and Sigma porcine pancreatic lipase. A hydrocarbon internal standard permitted an evaluation of conversion to 1-octanol ester, from which was calculated a value for reactivity in μmol ester formed, min^{-1}, g^{-1}. Reactivities of ethyl, isopropenyl and 2,2,2-trichloroethyl esters were similarly calculated and the results tabulated as reactivity relative to the corresponding acid as unity (Table 1). A typical chromatogram is given in Figure 1. Under these conditions,

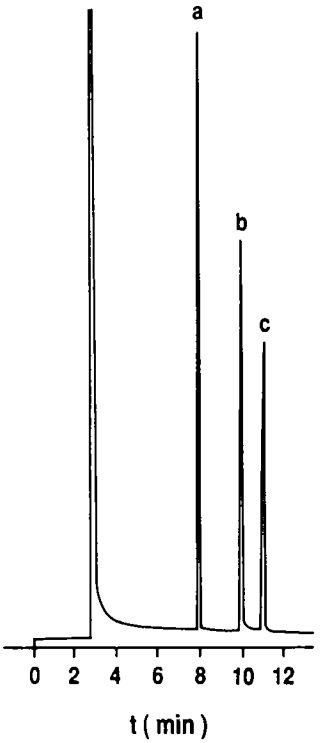

FIG. 1. Gas Chromatogram of a) 2-octanol octanoate, b) 1-octanol octanoate and c) octadecane, in a molar ratio of 1.765:1.797:1.000, using the SPB-1 column at 200°C. Similar chromatograms were used as standards to monitor lipase esterifications.

TABLE 1

Relative Rate of Esterification of 1-Octanol in Hexane[a]

Enzyme	Acid residue	Acid[b]	Ethyl[c]	Isopropenyl[c]	TCE[d]	SH[e]
C. rugosa	Acetyl	1.00	—[f]	19.8	23.4	15.3
	Octanoyl	1.00	—	1.7	8.5	ND
	Oleoyl	1.00	—	1.5	3.5	ND
A. niger	Acetyl	1.00	0.071	1.96	0.41	5.86
	Octanoyl	1.00	0.038	0.11	0.067	ND
	Oleoyl	1.00	<0.04	0.39	<0.04	ND
M. miehei	Acetyl	1.00	0.30	0.34	0.25	1.5
	Octanoyl	1.00	0.28	0.016	0.23	ND
	Oleoyl	1.00	0.27	0.29	0.27	ND
Pancreatic	Acetyl	1.00	0.32	0.62	2.07	2.57
	Octanoyl	1.00	<0.01	0.12	0.23	ND
	Oleoyl	1.00	—[f]	0.56	<0.15	ND

[a]See Materials and Methods. Reactions were duplicated and GLC analyses were reproducibly ± 3%. Activities presented are relative to the acid as 1.00; actual values obtained for free acids are: (acid, activity in μmol ester formed, min^{-1}, g^{-1}) for *C. rugosa* (acetic, 0.058; octanoic, 1.25; oleic, 1.01), *A. niger* (acetic, 2.66; octanoic, 20.6; oleic, 10.3), *M. miehei* (acetic, 4.91; octanoic, 250; oleic, 310), and pancreatic (acetic, 0.32, octanoic, 8.4; oleic, 5.4).

[b]Free acid.

[c]Ester.

[d]2,2,2-Trichloroethyl ester.

[e]Thiolacetic acid.

[f]Very slow (less than 0.01).

ND, not determined.

TABLE 2

Activity of Lipases in Various Organic Solvents (1-Octanol and Octanoic Acid)[a]

Enzyme	Hexane	Toluene	Ether	DME[b]	M[c]	Acetone
M. miehei	300	150	4.3 (260)	—[d] (100)	8.0 (220)	0.3 (250)
A. niger	20.6	2.5 (8.5)	—[d]	— (-)	—[d] (2.3)	— (0.77)
C. rugosa	1.25	—[d] (0.56)	— (0.77)	— (-)	— (0.083)	— (-)
Pancreatic	8.4	4.6	0.21 (3.4)	— (2.4)	0.55 (5.8)	0.20 (5.9)

[a]See Materials and Methods. Reactions were duplicated and GLC analyses were reproducibly ±3%. Activities are in units of μmol of ester formed, min^{-1}, g^{-1}. Figures in parentheses are activities of recovered enzyme measured in hexane.

[b]1,2-Dimethoxyethane.

[c]Methylene chloride.

[d]Less than 0.01.

the ethyl esters were uniformly less reactive than the corresponding free acids. Because the carbonyl groups of the isopropenyl and 2,2,2-trichloroethyl esters render the ester carbonyls electron deficient, however, one might expect that the lipases would be more readily acylated. In fact, the "activated" esters were indeed more reactive with *C. rugosa* lipase, especially the acetates. But these esters were generally less reactive than the free acids using the other lipases. To the degree that an acylated enzyme has no memory of the source of the acyl residue, these relative reactivities suggest that carbonyl polarity in the substrate bears little relationship to the ease of enzyme acylation. It is interesting to note that thiolacetic acid was more reactive than acetic acid. The longer chain acids are favored by the enzymes over acetic acid, as previously established. The counterunit (alkoxy group for an ester) is sometimes more effective in promoting reaction of the shorter acid, perhaps because acetic acid tends to self-associate in hexane, although its esters are monomeric and more hydrophobic.

The reactivity of enzymes in organic media has been reviewed by Klibanov (14), and it is clear that solvent can profoundly influence both stability and reactivity. We conducted esterifications of octanoic acid with 1-octanol, using the same lipase preparations in various organic solvents for 1 hr, and determined reactivities as before. The lipase from such reactions was recovered and used for the same esterification reaction in hexane, in order to determine residual lipase activity (Table 2). In each case, the esterification reaction was fastest in hexane, about

half as rapid in toluene for the lipases of *M. miehei* and porcine pancreas, and very slow otherwise. Evaluations of recovered activity often showed significant losses particularly for *A. niger* and *C. rugosa* lipases, especially in 1,2-dimethoxyethane. The loss in activity for the two aforementioned lipases in acetone is interesting, because that solvent is often used to precipitate crude protein preparations. An exact determination of the nature of these losses in each case is beyond the scope of this investigation, but it is apparent that reactivity in, and recovery of enzyme activity from, organic solvents would have to be uniquely determined and optimized for a particular reaction, enzyme and solvent.

Esterification of 2-octanol. The same lipase preparations were used to catalyze the esterification of 2-octanol with octanoic acid in hexane at 30 ± 0.2°C. Acetic acid and its esters reacted too slowly, and octanoic acid was selected, because the results obtained for octanoic acid were expected to be similar to those that would be obtained for longer chain acids (12). The degree of conversion was obtained by GC (Fig. 1), and the stereobias (enantiomeric excess) in the unreacted 2-octanol was determined by conversion to diastereomeric carbamates with (S)-α-methylbenzylisocyanate that then could be analyzed by GC (Fig. 2). These data permitted the calculation of enantiomeric ratio (E_R), a number that expresses the relative reactivity of enantiomers with an enzyme (15) (Table 3). Although the derivation for E_R was based on pure enzyme, i.e., a single enzymatically active species, a mixture of enzymes such as an impure commercial preparation would, at most, cause the calculated E_R to drift, particularly at greater conversion. Conversions were less than 50% and are offered as a rough indicator of relative reactivity of the enantiomers.

As reported previously, the stereoselection of *M. miehei* lipase was high for octanoic acid; it is also high for the isopropenyl and 2,2,2-trichloroethyl esters. The Amano-Lipase K *(A. niger)* also was high enough to be useful and we partially resolved 2-hexadecanol to show the utility of such a procedure. Previous evaluation of this lipase (12) used the commercial powder that had low activity. Deposition of the *A. niger* lipase powder on Duolite, however, markedly increased its activity and allowed a more accurate determination of its stereobias in this reaction. The increased activity of the immobilized *A. niger* lipase is probably due to improved stability of the enzyme (16). The immobilization process frequently acts to reduce one or another of the denaturation processes, and the assay method that is conducted during a time interval leads to a higher calculated specific activity than did the original enzyme powder. The immobilized *A. niger* lipase was allowed to catalyze the esterification of 2-octanol with oleic acid. At 45% conversion, the residual alcohol was 85% S:15% R (70% ee:S), and the product oleate ester was 87% R (Fig. 2). The E_R would be ca. 29, and a conversion in the vicinity of 58% would assure a pure (S)-2-hexadecanol (17). The *C. rugosa* showed no bias with the octanoic acid and low, opposite biases with the two octanoyl esters. Enzyme recovered from reaction of the isopropenyl ester was used to esterify octanoic acid (no bias for octanoic acid), then recovered and used for the isopropenyl ester (same result for the ester). Thus, the alteration in selectivity is real and reproducible. More striking is the modest bias shown by pancreatic lipase with octanoic acid, and the very strong selectivity with the 2,2,2-trichloroethyl ester. Recently, Deleuze et al. (10) reported

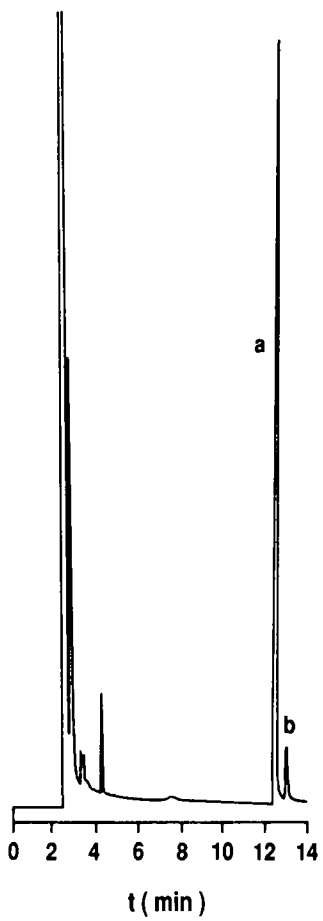

FIG. 2. GLC analysis of α-methylbenzyl carbamates obtained by the reaction of 2-hexadecanol recovered from its oleate ester. The ester had been formed from alcohol and acid using *A. niger* lipase (Amano-Lipase K) on Duolite. Analysis uses an SPB-1 column at 280°C; a) is the R,S-diastereomer (R-alcohol) and b) is the S,S-diastereomer.

TABLE 3

Stereoselectivities of Lipases in Octanoylation of 2-Octanol in Hexane[a]

Enzyme	Acid	Isopropenyl[b]	TCE[c]
C. rugosa	1.0	1.3[d]	1.3
A. niger	>40[e]	>40[e]	>40[e]
M. miehei	>40	>40	>40
Pancreatic	9.6	—[f]	>40

[a] See Results and Discussion for selectivity measurement. The (R)-2-octanol reacts fastest, except as noted.

[b] Ester.

[c] 2,2,2-Trichloroethyl ester.

[d] The (S)-2-octanol reacts slightly faster.

[e] Although calculation gives an exact figure, the analytical technique is insufficiently precise and the error in calculated value increases with higher relative rates. For practical purposes selectivities greater than 40:1 are likely to be useful in kinetic resolution.

[f] Reaction too slow.

the results of competition experiments showing that the relative reactivity of pairs of esters with lipases was independent of the alcohol that was being esterified. Likewise, the relative reactivity of pairs of alcohols with acylated lipases was independent of the source (e.g., structure of the alcohol component of the ester) of acid residue. These observations are consistent with a generally accepted view that lipases first become acylated, and then undergo reaction with an available nucleophile in a discrete second phase of operation. Implicit is an assumption that the nonacyl component of the substrate is shed from the enzyme into the medium, where it becomes a minor component in the pool of available nucleophiles. Our observations do not confound this picture, but merely indicate that substrates and nucleophiles can, perhaps by virture of alternate associations with the protein and their overwhelming presence (compared to enzyme concentration), alter somewhat the delicate balance of stereoselectivity of an acylated enzyme. Similar observations were reported earlier, for example, wherein stereoselectivity involving the esterification of *rac*-2-octanol with octanoic acid using *M. miehei* lipase was reduced significantly in the presence of the much less reactive acetic acid, though the same reaction proceeded at a slower rate with no change in stereoselectivity in the presence of unreactive phenyl(trifluoromethyl)carbinol (18).

Lipase selectivity. Much interest exists in exploring the selectivities of lipases for projected industrial reactions (3–6), and a number of patents have been issued that are based on preferential reactions of various lipases with fatty acids and their derivatives (19). Given the problem of selectively converting one reagent present in a mixture of potential reagents to a product, one is faced essentially with a kinetic problem. With sufficient reaction time, a product mixture would be obtained that is at equilibrium thermodynamically. Therefore, how selective must a catalyst be to be useful?

Competitive reactions were conducted that were chosen based on the data of Table 1. Isopropenyl heptanoate and ethyl octanoate were allowed to transesterify 1-octanol competitively, using *C. rugosa* lipase on Duolite in hexane. Because isopropenyl esters are better substrates for this lipase than ethyl esters and will react irreversibly, one expects to see faster and more complete conversion to octyl heptanoate than to octyl octanoate. The data (Fig. 3) show the general predicament inherent in the use of enzymes as catalysts, when selectivity is modest. The heptanoate product contains only ~2% of the octanoate ester, if the reaction is terminated at ~19% conversion (5 hr). However, at 86% conversion (144 hr), the octanoate component is >17% of the product. In a related experiment that would yield the same two esters as products, but should lead to a mixture richer in octanoate ester (Fig. 4), isopropenyl heptanoate and 2,2,2-trichloroethyl octanoate were allowed to react with 1-octanol. In this case, it was expected that octyl octanoate ester would form more quickly. Indeed, this was so, and then the octyl heptanoate ester began to form. Eventually *trichloroethyl heptanoate* also was observed in the reacting mixture, indicating that trichloroethyl esters do react reversibly.

In summary, several acids and their esters have been evaluated in esterification reactions of 1- and 2-octanol as typical aliphatic alcohols, using several commercially available lipases. Reactions were fastest in hexane, as compared with several other common organic solvents,

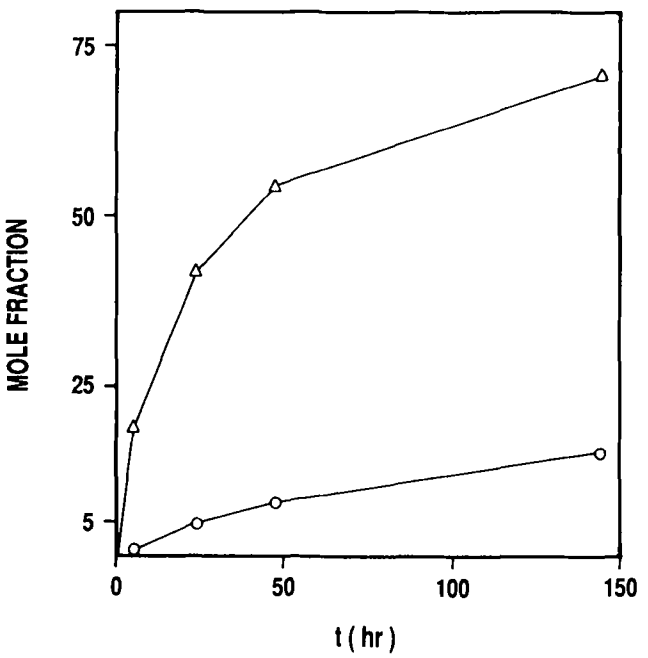

FIG. 3. Reaction of isopropenyl heptanoate (0.01 M), ethyl octanoate (0.10 M) and 1-octanol (0.12 M) in wet hexane with *C. rugosa* lipase (0.300 g/mmol of ester) at 30°C. Δ:1-octanol heptanoate; 0:1-octanol octanoate.

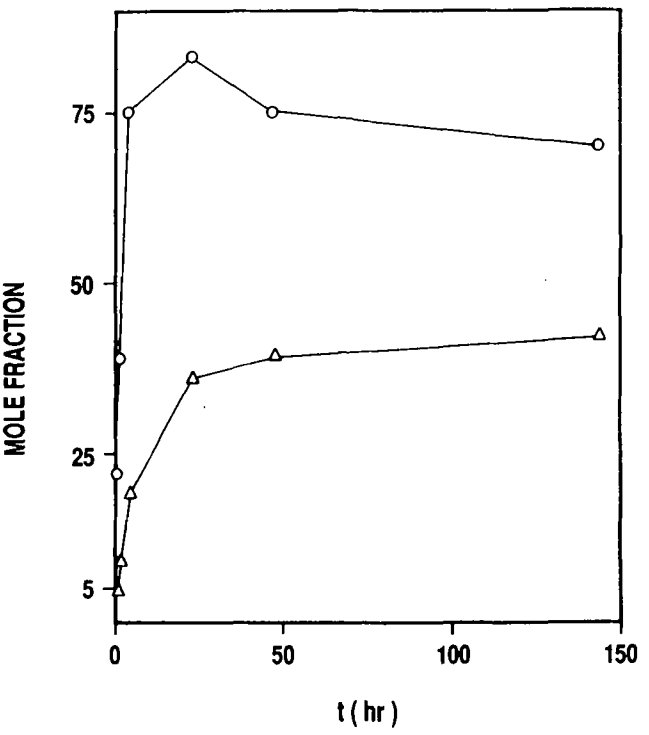

FIG. 4. Reaction of isopropenyl heptanoate (0.10 M), 2,2,2-trichloroethyl octanoate (0.10 M) 1-octanol (0.12 M) in wet hexane with *M. miehei* lipase (0.025 g/mmol of ester) at 30°C. Δ:1-octanol heptanoate; 0:1-octanol octanoate.

and the organic acid itself is usually the best substrate. An exception to this involves the reactions of *C. rugosa* lipase, which reacts more rapidly in hexane with isopropenyl and 2,2,2-trichloroethyl esters. Further evaluation of lipase selectivity using *rac*-2-octanol indicated a previously unobserved high stereoselectivity for the *A. niger* lipase and, unexpectedly, variation in stereobias with source of acyl residue using the lipases of *C. rugosa* and porcine pancreas. Exemplifications of lipase selectivity, based on relative reactivities of esters (Table 1), demonstrate the relationship between selectivity and product purity. Applications of lipase selectivities (as in kinetic resolution of enantiomers [16]) are best applied in the sense of purifying a starting material. Only if the selectivity of a lipase is such, to make the catalyst virtually specific, can it be used to yield a nearly pure product.

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REFERENCES

1. Macrae, R.R. (1983) in *Microbial Enzymes and Biotechnology* (Fogarty, W.M., ed.) pp. 225-250, Applied Science Publishers, New York, NY.
2. Iwai, M., and Tsujisaki, Y. (1984) in *Lipases* (Borgstrom, B., and Brockman, H.L., eds.) pp. 443-469, Elsevier, Amsterdam, The Netherlands.
3. Werdelmann, B.W., and Schmid, R.D. (1982) *Fette Seifen Anstrichm. 84,* 436-443.
4. Neidelman, S.L., and Geigert, J. (1984) *J. Am. Oil Chem. Soc. 61,* 290-297.
5. Posorske, L.H. (1984) *J. Am. Oil Chem. Soc. 61,* 1758-1760.
6. Nielsen, T. (1985) *Fette Seifen Anstrichm. 87,* 15-19.
7. Kirchner, G., Scollar, M.P., and Klibanov, A.M. (1985) *J. Am. Chem. Soc. 107,* 7072-7076.
8. Sweers, H.M., and Wong, C.-H. (1986) *J. Am. Chem. Soc. 108,* 6421-6422.
9. Degueil-Castaing, M., DeJeso, B., Drouillard, S., and Maillard, B. (1987) *Tetrahedron Lett. 28,* 953-954.
10. Deleuze, H., Langrand, G., Millet, H., Barratti, J., Buono, G., and Triantaphylides, C. (1987) *Biochim. Biophys. Acta 911,* 117-120.
11. Rothman, E.S., Serota, S., Perlstein, T., and Swern, D. (1962) *J. Org. Chem. 27,* 3123-3126.
12. Sonnet, P.E., and Baillargeon, M.W. (1987) *J. Chem. Ecol. 13,* 1279-1292.
13. Pirkle, W.H., and Hauske, J.R. (1977) *J. Org. Chem. 42,* 2781-2782.
14. Klibanov, A.M. (1986) in *Protein Engineering, Applications in Science, Medicine, and Industry,* pp. 341-349, Academic Press, New York, NY.
15. Wang, Y.-F., Chen, C.-S. Girdaukas, G., and Sih, C.J. (1984) *J. Am. Chem. Soc. 106,* 3695-3696.
16. Lavayre, J., and Baratti, J. (1982) *Biotechnol. Bioeng. 24,* 1007-1013.
17. Martin, V.S., Woodard, S.S., Katsuki, T., Yamada, Y., Ikeda, M., and Sharpless, K.B. (1981) *J. Am. Chem. Soc. 103,* 6237-6240.
18. Sonnet, P.E. (1987) *J. Org. Chem. 52,* 3477-3479.
19. Sonnet, P.E. (1987) in *Oleochemicals: Fatty Acids, Fatty Alcohols, and Fatty Amines,* Educational Committee of the American Oil Chemists' Society, Champaign, IL.

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Interaction of Rabbit Lipoproteins and Red Blood Cells With Liposomes of Egg Yolk Phospholipids

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After intravenous injection of liposomes prepared from egg yolk phospholipids into rabbits, the phospholipids were readily assimilated by the lipoproteins, and there were increases in the circulating levels of cholesterol and phospholipids. The increases in cholesterol level were mainly due to increases of free cholesterol. Gradient ultracentrifugation showed that the lipoproteins decreased in density, and gel filtration chromatography showed that they increased in particle size. Upon electrophoresis, they exhibited slower mobility. Liposomes recovered from rabbits 3 hr after the injection contained free cholesterol, apolipoproteins A-I, E and traces of C. The apolipoprotein may target the liposomes for uptake by hepatocytes. Incubation of the liposomes with rabbit red blood cell membranes in vitro caused a decrease in cholesterol content of the membranes. However, the cholesterol/phosphate ratio in red blood cells isolated from the rabbits after the injection of liposomes did not change significantly, suggesting rapid replenishment of red blood cell cholesterol in vivo, possibly by equilibration with lipoprotein cholesterol or tissue cholesterol. These results suggest that the injection of phospholipid liposomes may have an antiatherogenic effect by the removal of tissue cholesterol and enhancing hepatic disposal of cholesterol through the reverse cholesterol transport mechanism. *Lipids 23,* **961-967 (1988).**

The antiatherogenic effect of intravenous injections of phospholipids has been demonstrated in several experimental animal models. Friedman et al. (1) first reported that the infusion of a suspension of animal lecithin affected a marked resolution of atherosclerotic infiltration and reduced cholesterol deposition in the aorta of rabbits in which atherosclerosis had been induced by a diet containing 1% or 3% cholesterol. These observations were confirmed by others (2,3). Regression of atherosclerotic lesions in baboons after the injection of phospholipids was reported by Howard et al. (4), and similar observations in Japanese quails were reported by Stafford and Day (5).

Recent studies by Williams and Scanu (6) in dogs demonstrated the uptake of apolipoprotein A-I (apo A-I) and cholesterol by intravenously injected phospholipid liposomes, with concomitant uptake of phospholipids by high density lipoprotein (HDL). The authors suggested that the injected liposomes mobilized tissue cholesterol. A possible antiatherogenic mechanism of injected phospholipid liposomes was proposed by Williams et al. (7), based on their observation of liposomes injected into hypercholesterolemic rabbits. They found that the liposomes acquired apolipoproteins E (apo E) from beta-very low density lipoprotein (β-VLDL), and the apo E containing liposomes then competed with β-VLDL for binding to apo E receptors on macrophages, thus, diminishing the uptake of cholesteryl esters from β-VLDL by the macrophages.

To seek further explanation of the antiatherogenic effect of phospholipid liposomes, we injected liposomes of egg yolk phospholipids into normolipidemic rabbits, to assess their effects on cholesterol transport. We found extensive interaction between the liposomes and serum lipoproteins. The injected phospholipids were readily assimilated by serum lipoproteins and the liposomes recovered from rabbit blood contained, in addition to cholesterol, apolipoproteins A-I, E and traces of C. We also examined the effect of the liposomes on cholesterol and phospholipid contents of red blood cells. Taken together, our data support the contention that intravenous injection of phospholipid liposomes may mobilize tissue cholesterol and enhance the reverse cholesterol transport mechanism, thus having an antiatherogenic effect.

MATERIALS AND METHODS

Rabbits. Male New Zealand white rabbits, ca. six months of age, were fed normal rabbit chow and maintained at controlled temperature with a 12-hr dark/light cycle, by the Division of Veterinary Resources at the University of Miami School of Medicine. Food was removed from the rabbits at least 12 hr prior to collection of blood samples or injection of liposomes through the peripheral ear vein.

Liposomes. Injection grade egg yolk phospholipids (lot #VH001V) were a generous gift of Asahi Chemicals (Tokyo, Japan), and contained 98.8% phosphatides, of which 71.0% was phosphatidylcholine; 20.2%, phosphatidylethanolamine; and less than 1%, lysophosphatides. Liposomes were prepared by sonication according to Huang (8). Briefly, phospholipids were suspended in 0.15 M NaCl, pH 7.4, at a concentration of 300 mg/ml, and sonicated for three 10-min intervals at 0°C, with the standard tip of the sonicator (Heat Systems Ultrasonics, Plainview, NY) at a setting of 50 W. The sonicated lipids were then centrifuged at 120,000 × *g* for 30 min to remove Ti particles and nonliposomal lipids. The supernatant was collected and passed through a 0.45 μm pore filter. Liposomes were sized by gel permeation chromatography on BioGel A-5m (BioRad Laboratories, Richmond, CA) using a 1 × 90 cm column. Typical preparations of liposomes contained >90% of the phospholipids in a major peak which corresponded to smooth unilamellar vesicles as previously described (8). The remaining phospholipids that eluted near the void volume were probably composed of multilamelar vesicles and other large lipid aggregates.

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Abbreviations: Apo A-I, apolipoprotein A-I; apo E, apolipoprotein E; (β-)(V)LDL, (beta-)(very) low density lipoprotein; HDL, high density lipoprotein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

Lipid assays. Cholesterol in serum and serum fractions was determined by the cholesterol oxidase method of Allain et al. (9). Free cholesterol was determined using the same method with the omission of cholesteryl esterase from the reagent.

Triglycerides in rabbit serum were determined using a commercially available kit (Craig Bioproducts Inc., Streamwood, IL) based on the method of McGowan et al. (10).

Serum phospholipids were estimated after precipitation of lipoproteins with 100 volumes of 10% trichloroacetic acid as described by Zilversmit and Davis (11). Phospholipids were determined as inorganic phosphorous after digestion with perchloric acid at 170–180°C for ca. 30 min by the method of Bartlett (12).

Agarose gel electrophoresis. Agarose gel electrophoresis of serum lipoproteins was performed using the Corning (Palo Alto, CA) ACI electrophoresis system according to the manufacturer's procedures. Lipoproteins were stained with Fat Red 7B, and gels were scanned at 510 nm with a Corning model 730 densitometer.

Density gradient ultracentrifugation. Density gradients were prepared by the single spin method of Terpstra et al. (13) with minor modifications. Two ml of serum was adjusted to density 1.21 g/ml by the addition of 0.809 g KBr and 0.050 g sucrose, and placed at the bottom of an ultracentrifuge tube and carefully overlayered with the following KBr solutions: 2 ml d = 1.20 g/ml, 4 ml d = 1.10 g/ml and 4 ml d = 1.006 g/ml. Ultracentrifugation was performed in a SW 40Ti rotor (Beckman Instruments, Palo Alto, CA) at 39,000 rpm using a L5-65 ultracentrifuge (Beckman) for 22 hr at 18°C. Gradients were fractionated using an ISCO (Lincoln, NE) density gradient fractionator, and 0.75-ml fractions were collected.

Gel permeation chromatography. Columns (1.5 × 90 cm) were packed with BioGel A-5m equilibrated in 0.01 M Tris, pH 7.4, 0.15 M NaCl, 0.01% EDTA and 0.02% NaN_3 and kept at 4°C. A flow rate of 6 ml/hr was maintained with the use of a peristaltic pump, and 4-ml fractions were collected.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was according to O'Farrel et al. (14), using the discontinuous buffer system of Laemmli (15) in 12% polyacrylamide slab gels. Prior to analysis, samples were delipidated as above and dissolved in 0.01 M Tris, 1 mM EDTA, 1% SDS and 5% β-mercaptoethanol, pH 8.0. Gels were stained with 0.2% Coomassie Bright Blue in methanol/water/acetic acid (5:5:1, v/v/v) and destained in the same solution without dye until a clear background was obtained. Isoelectric focusing (IEF) was performed according to the method of Menzel et al. (16) with minor modifications. A 7.5% polyacrylamide gel containing 1.6% ampholines (Pharmalyte pH 4.5–6.5, Pharmacia Fine Chemicals, Piscataway, NJ) and 5.2 M urea was used. The anode chamber contained 0.01 M H_3PO_4 and the cathode chamber, 0.02 N NaOH. Samples were delipidated with ethanol/ether (3:1, v/v) according to Scanu and Edelstein (17), and dissolved in 0.01 M Tris-HCl, pH 8.2, containing 1% SDS, 2% ampholine, 5% β-mercaptoethanol and 13% sucrose. Electrophoresis was performed at 4°C at 200 V, until the current was zero.

Preparation of red blood cell ghosts. Red blood cell ghosts were prepared by the method of Dodge et al. (18). Blood was collected in tubes containing EDTA (final concentraton 2 mg/ml), and subjected to centrifugation at 1,000 × *g* for 15 min. After removal of the plasma and the buffy coat, the red cells were washed 3 times with 0.15 M NaCl, 0.01 M Tris, pH 7.5. Hemolysis was effected by the addition of 20 volumes of 0.01 M Tris, pH 7.5, with gentle mixing. Red cell ghosts were sedimented by centrifugation at 20,000 × *g* for 45 min, washed 5 times and resuspended in 0.15 M NaCl, 0.01 M Tris, pH 7.5.

RESULTS

Changes in serum lipid levels. Each of eight rabbits was given an IV injection of egg yolk phospholipid liposomes at the dose of 60 mg phospholipid/kg animal weight. There was an immediate rise of serum phospholipid concentration (ca. 2-fold), which was sustained for about 1 hr. The concentration then began to decline, reaching a plateau above baseline 6–8 hr post-injection, and the elevation persisted for at least 72 hr. Approximately 15 min following injection, serum cholesterol levels began to rise, predominantly due to increases in free cholesterol, reaching peak levels between 3 and 4 hr. By the eighth hour, the cholesterol levels reached a plateau above the baseline level in five of the eight animals. Increases in cholesteryl ester concentrations lagged behind increases of free cholesterol by ca. 1 hr and were smaller in magnitude (0–12% above baseline). Serum triglyceride levels varied among the animals. In control animals injected with saline, similar variations in triglyceride levels were observed, indicating that the triglyceride changes could not be attributed to the liposomes. Figure 1 summarizes serum lipid levels of eight rabbits before and 4 hr after liposome injection.

Changes in serum lipoproteins—Agarose gel electrophoresis. Figure 2 shows the results of agarose gel electrophoresis of serum samples before and after liposome injection. Prior to injection (Panel A), the peaks of LDL (beta mobility) and VLDL (pre-beta mobility) are merged, and HDL (alpha mobility) appears as a separate peak. Upon electrophoresis, liposomes gave a single peak with slower migration than LDL (data not shown). Thirty min after injection, two major peaks of lipid-staining material were observable (Panel B) with slower mobilities than the

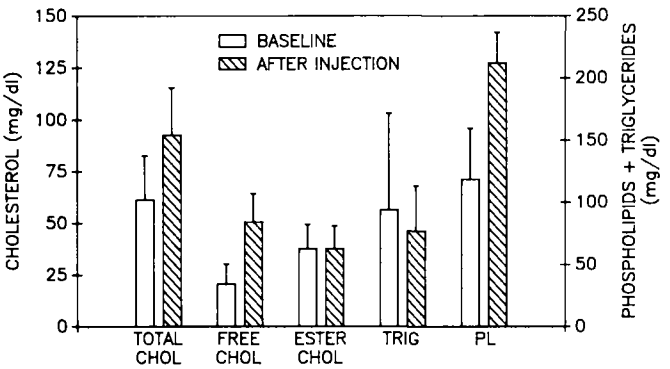

FIG. 1. Changes in rabbit serum lipid levels after injection of egg yolk phospholipid liposomes. Eight rabbits were each injected with egg yolk phospholipid liposomes (60 mg/kg wt). Serum was obtained from blood samples taken before and 4 hr after injection. CHOL, cholesterol; TRIG, triglycerides; PL, phospholipids.

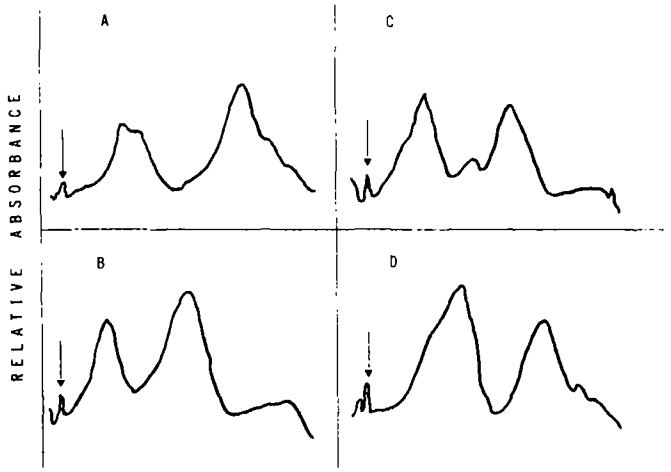

FIG. 2. Agarose gel electrophoresis of rabbit serum. Serum was obtained from blood samples taken before and after injection of egg yolk phospholipid liposomes and subjected to agarose gel electrophoresis. Lipoproteins were stained with Fat Red 7B, and the gels scanned at 510 nm. The origin is marked with an arrow. The cathode is to the left and the anode to the right of the panel. A, before injection; B, 30 min after injection; C, 4 hr after injection; D, 48 hr after injection.

peaks obtained from control serum (Panel A). The first peak from the left represents liposomes in addition to VLDL and LDL, and the second peak corresponds to HDL. Three peaks are observed in Panel C (4 hr after injection). The first one with slower mobility than control LDL contains VLDL, LDL and liposomes, and the large peak to the right represents HDL. Its mobility is faster than that in Panel B, but slower than that in Panel A, indicating partial restoration of HDL. In addition, a small peak is observed between the two major peaks. This peak represents a component of the altered HDL, which was slow to undergo restoration. The electrophoretic pattern of HDL in Panel D (48 hr after injection) is essentially the same as before injection, but a larger pre-beta peak persists. In Panels B, C and D, the liposome and LDL peaks are not distinguishable. However, in control experiments where liposomes and control serum were mixed together and then subjected to agarose gel electrophoresis, the LDL and liposome peaks were separated, although not completely resolved. These data indicate that the LDL peak had lower mobility after injection of liposomes. In addition, electrophoresis of serum mixed with liposomes did not reveal any alteration in the mobility of HDL, indicating that the changes observed in serum after injection were due to changes in the HDL particles and were not an artifact of electrophoresis, resulting from the presence of liposomes in the sample.

Density gradient ultracentrifugation. Serum samples were obtained prior to, 15 min after and 3 hr after injection of 200 mg phospholipid into a rabbit, and subjected to density gradient ultracentrifugation. VLDL at the top of the gradient was removed. Cholesterol and phospholipid contents of the gradient fractions were determined and the results are shown in Figure 3. Panel A shows the results of serum fractions obtained prior to injection, and two distinct peaks of cholesterol and phospholipids, corresponding to LDL and HDL, can be seen with maxima at fractions 4 and 11, respectively. The HDL peak shows

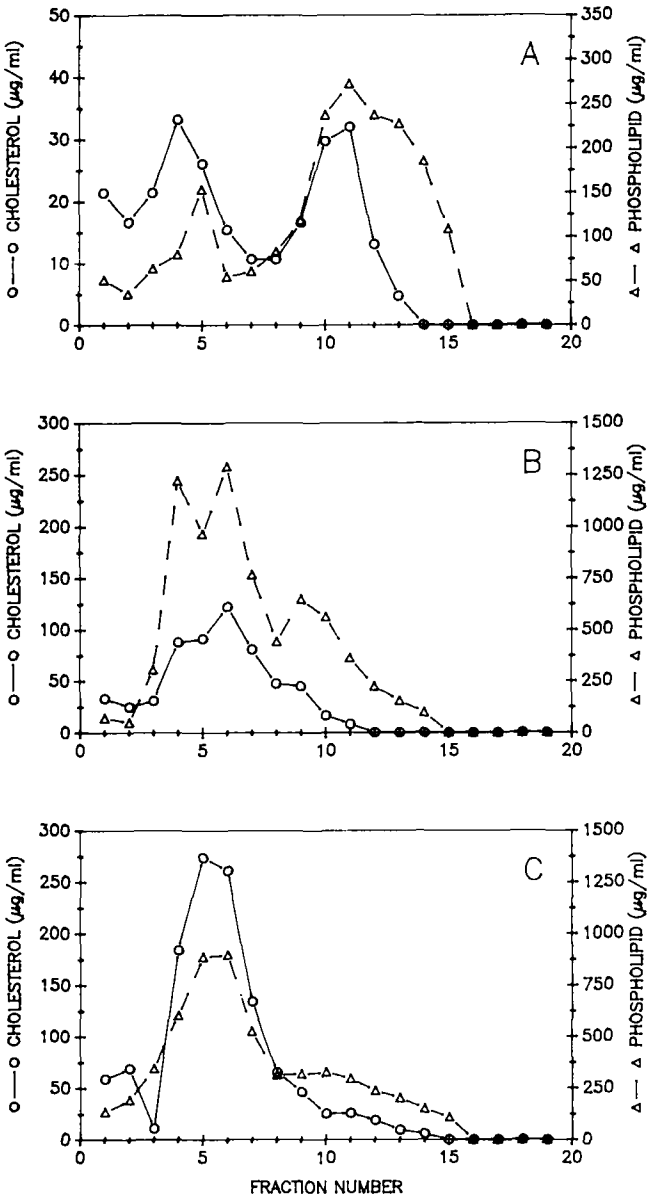

FIG. 3. Density gradient ultracentrifugation profile of rabbit serum. Two ml of serum was subjected to density gradient ultracentrifugation as described in Methods. Fractions of 0.75 ml were collected after tube piercing and displacement of the contents with a dense chase solution. Cholesterol and phospholipid contents of each fraction were assayed. Gradient density increases from left to right. A, before injection, serum cholesterol 28 mg/dl and serum phospholipid 55 mg/dl; B, 15 min after injection, serum cholesterol 42 mg/dl and serum phospholipid 219 mg/dl; C, 3 hr after injection, serum cholesterol 78 mg/dl and serum phospholipid 184 mg/dl.

heterogeneity in the distribution of cholesterol and phospholipids, the less dense fractions having greater contents of cholesterol relative to phospholipids. Panel B shows increases in phospholipids and cholesterol in all fractions, as well as a shift of the HDL peak to lower densities 15 min after injection. In a separate experiment, liposomes were subjected to density ultracentrifugation and were recovered between fractions 2 and 6, with the peak at fraction 3. The increase of phospholipids in the LDL region in Panel B appears to be due to superimposition of the liposomes. In Panel C (3 hr after injection of

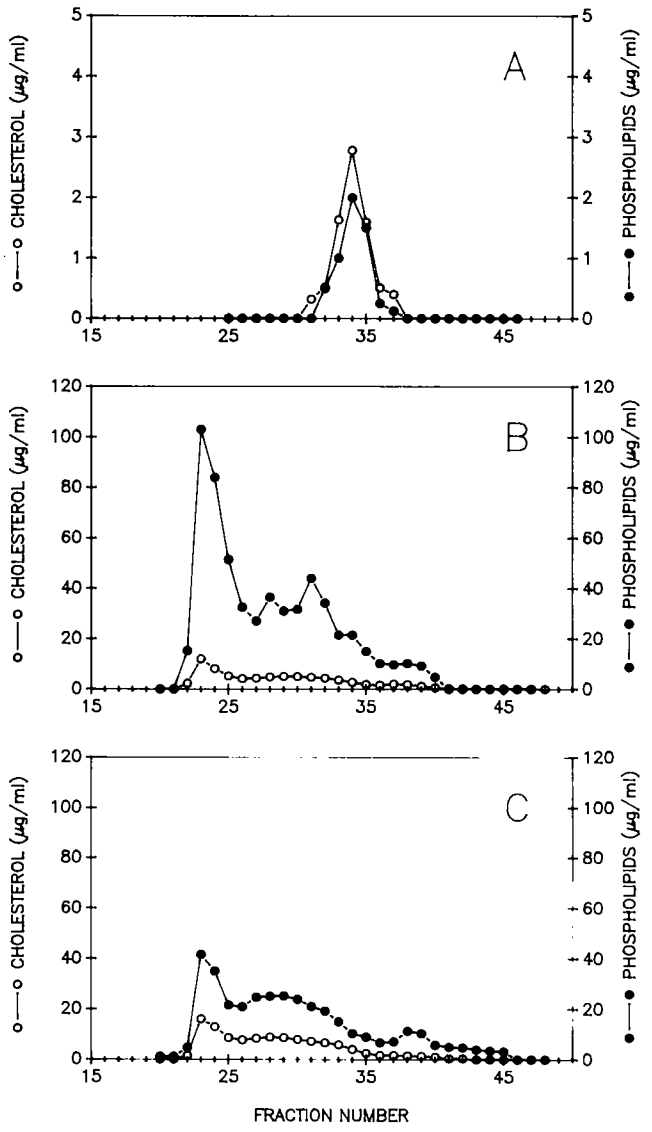

FIG. 4. Gel permeation chromatography of LDL and liposomes isolated by density gradient ultracentrifugation. Density gradient fractions 3 to 7 (Fig. 3) were combined and subjected to chromatography on BioGel A-5m, using a 1.5 × 90 cm column at a flow rate of 6 ml/hr. Fractions of 3 ml were collected and assayed for cholesterol and phospholipid contents. A, density gradient fractions 3 to 7, before injection; B, density gradient fractions 3 to 7, 15 min after injection; C, density gradient fractions 3 to 7, 3 hr after injection.

the liposomes), there is a major peak of phospholipids and cholesterol in the region of LDL. In both regions of LDL and HDL, increases of cholesterol and decreases of phospholipids are observable.

Gel permeation chromatography. Peak fractions from the density gradient ultracentrifugation (Fig. 3) were pooled and further analyzed by gel permeation chromatography to assess particle size and determine contents of cholesterol and phospholipids. Figure 4 shows the profiles of gel permeation chromatography of density gradient fractions 3-7 of Figure 3, corresponding to LDL in control serum. Panel A (control sample) shows only a single peak of cholesterol and phospholipids. Panel B reveals several peaks containing both cholesterol and phospholipids, 15 min after injection. The first peak from the left elutes in the region of control liposomes, and the second is a broad peak encompassing LDL and larger particles. Panel C (3 hr after injection) is qualitatively similar to the profile in Panel B, except for the obvious decrease in phospholipids and the appearance of a more-defined minor peak eluting in the region of control HDL. This peak indicates that a portion of HDL had acquired lower density to the range of LDL (Fractions 3-7, Fig. 3).

There was a decrease in the free cholesterol/phospholipid ratio in the LDL peak 15 min after injection, from 0.47 to 0.08, but an increase to 0.18, three hr after injection. Since the total amount of cholesterol in the LDL peak did not decrease significantly, or actually increased slightly after liposome injection, the decrease in this ratio reflects uptake of phospholipids from the liposomes by the LDL particles.

Similar studies were conducted with density gradient fractions 10 to 15 (HDL) of Figure 3. The results are shown in Figure 5. In each case there was a single peak containing both cholesterol and phospholipid. The rapid uptake of phospholipids by HDL 15 min after injection is evident in Panel B, and the loss of phospholipids thereafter is evident in Panel C.

Identification of liposome-associated apolipoproteins. For the isolation of liposomes from serum, serum samples were subjected to ultracentrifugation at a density of 1.006 g/ml to remove VLDL. The $d > 1.006$ g/ml fraction was then centrifuged at density 1.25 g/ml to separate the total lipoprotein fraction ($d < 1.25$ g/ml). This fraction was subjected to gel permeation chromatography for the separation of individual lipoproteins.

Figure 6 shows typical cholesterol and phospholipid profiles before and after injection of liposomes. Liposomes were recovered in the first peak eluted from the column (fractions 21-25). These fractions were pooled, lyophilized and delipidated. The protein pellet obtained was solubilized in the appropriate buffer (see Methods) and subjected to IEF. Figure 7 shows the liposomes recovered 3 hr after injection to contain the isoforms of apo E as the major protein and smaller amounts of apo A-I. The C apolipoproteins were also observed in trace amounts when gels were subjected to silver staining (data not shown). The isoforms of apolipoproteins E, A-I and C were verified by molecular weight estimations using two-dimensional electrophoresis with SDS-PAGE as the second dimension (data not shown).

Changes in red blood cells in vitro and in vivo. The increase in serum cholesterol found after the injection of liposomes could come from tissues including the arterial wall, the lipoproteins and possibly red blood cells. To test the ability of liposomes to remove cholesterol from red blood cells, we incubated red blood cell ghosts with liposomes and found decreases in the content of membrane cholesterol in a dose- and time-dependent manner (Figs. 8 and 9). Corresponding to the increase of cholesterol associated with the liposomes, was a decrease of cholesterol/phospholipid ratio in the cell membrane.

The above described decrease of cholesterol in red blood cell ghosts was not, however, observed in red blood cells in vivo. Three rabbits were each injected with liposomes (50 mg phospholipid/kg body weight) and blood was collected in EDTA (final concentration: 2 mg/ml blood). The hematocrit was measured and red blood cells were used

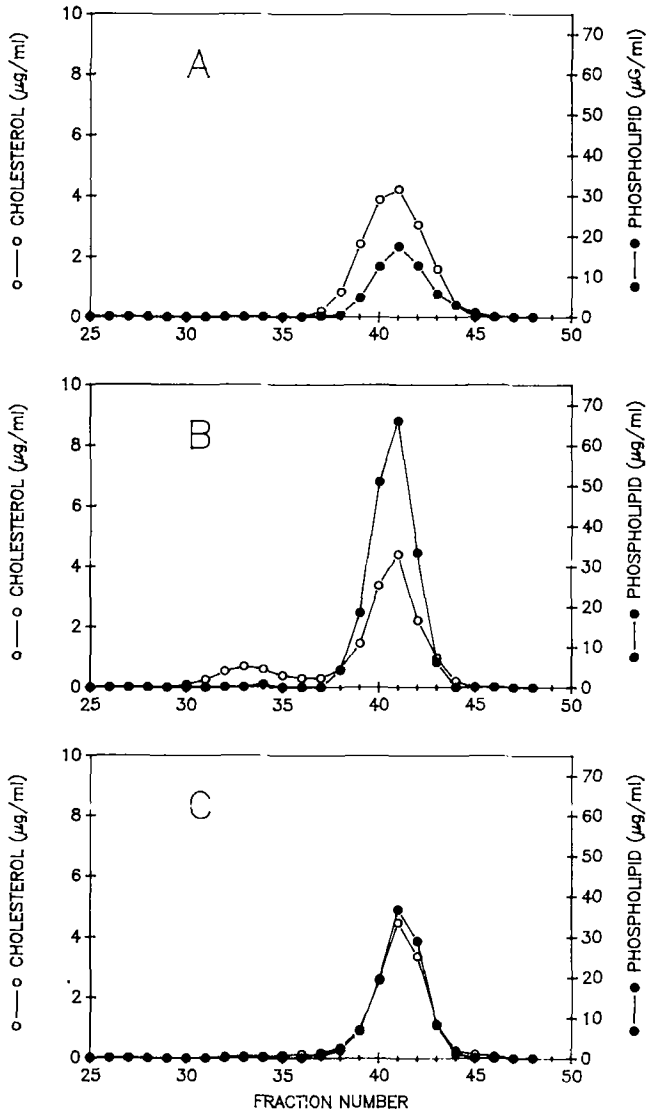

FIG. 5. Gel permeation chromatography of HDL isolated by density gradient ultracentrifugation. Density gradient fractions 10 to 15 (Fig. 3) and subjected to chromatography on BioGel A-5m using a 1.5 × 90 cm column at a flow rate of 6 ml/hr. Fractions of 3 ml were collected and assayed for cholesterol and phospholipid contents. A, density gradient fractions 10 to 15, before injection; B, density gradient fractions 10 to 15, 15 min after injection; C, density gradient fractions 10 to 15, 3 hr after injection.

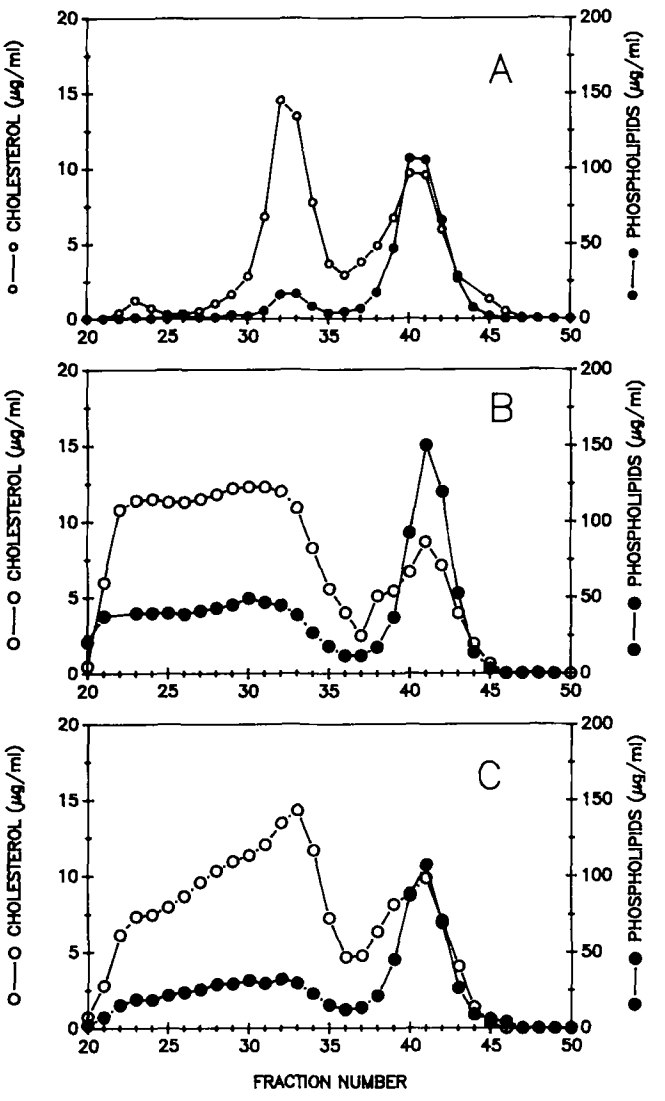

FIG. 6. Gel permeation chromatography of density 1.006 to 1.25 g/ml serum fractions before and after liposome injection. Serum samples were subjected to ultracentrifugation, and the density 1.006 to 1.25 g/ml fraction subjected to gel permeation chromatography on BioGel A-5m using a 1.5 × 90 cm column at a flow rate of 6 ml/hr. Fractions of 3 ml were collected and assayed for cholesterol and phospholipid contents. A, before injection; B, 50 min after injection; C, 3 hr after injection.

to prepare ghosts. The cholesterol and phospholipid contents of the red blood cell ghosts were determined and the cholesterol/phospholipid ratio calculated. Results in Table 1 show that the cholesterol/phospholipid ratio before and after injection did not change significantly, and the variations were within errors of assay (coefficient of variation for the same sample measured in pentuplicate was ±3.1%). Also, there was no appreciable change in the hematocrit value, indicating no loss of red blood cells resulting from the injection of liposomes.

DISCUSSION

Our data show that serum cholesterol and phospholipid levels increase after injection of egg yolk phospholipid liposomes (Fig. 1). Figures 2-5 indicate profound alterations of serum lipoproteins after the injection. There was a rapid assimilation of the injected phospholipids by the lipoproteins and, consequently, there were decreases in the density of LDL and HDL (Fig. 3), with increased particle sizes and decreased electrophoretic mobilities (Fig. 2). These results were qualitatively similar to results obtained after incubation of lipoproteins with liposomes in vitro (recently reviewed in Reference 19). It is of interest that the 2- to 3-fold increase in serum-free cholesterol was associated mainly with the liposomes (Figs. 4 and 6). Similar observations have been reported after infusion of egg yolk phospholipids into dogs (6), except that apo E was not identified in liposomes. It is not likely that the cholesterol taken up by the liposomes was newly

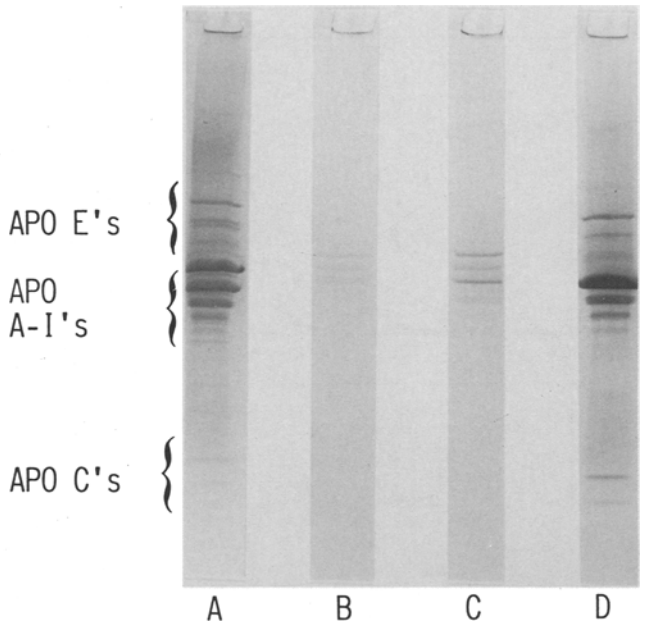

FIG. 7. Presence of apolipoproteins in recovered liposomes. The d > 1.006 to 1.25 g/ml fraction from serum was applied to a BioGel A-5m column as described for Fig. 6. Peak fractions were combined, and after concentration and delipidation were subjected to isoelectric focusing. A, HDL, before injection, column fractions 38–44; B, liposomes, 3 hr after injection, column fractions 21–25; C, liposomes, 3 hr after injection, column fractions 26–29; D, HDL, 3 hr after injection, column fractions 38–44.

synthesized, but was probably from the efflux of tissue cholesterol. Byers and Friedman (20) have shown in the hepatectomized rat that injection of phospholipids caused increased circulating cholesterol levels that could not be attributed to hepatic source.

Although incubation of red blood cell ghosts with liposomes caused removal of membrane cholesterol in vitro, no change in the cholesterol/phospholipid ratio in red blood cells was observed after injection of liposomes in vivo (Table 1). This discrepancy could be explained on the assumption that the cholesterol of red blood cells in vivo is replenished by rapid equilibration with cholesterol in lipoproteins and/or tissues. These data suggest that, if circulating red blood cells incorporate liposome phospholipids, they must also incorporate cholesterol derived from lipoproteins or tissues to maintain a steady cholesterol/phospholipid ratio.

It has been shown previously that incubation of phospholipid liposomes with fibroblasts in culture caused cellular efflux of cholesterol (21,22). In our experience, we found that the cholesterol content of human fibroblasts decreased as much as 30% by incubation with egg yolk phospholipid liposomes (unpublished observation). The data in Figure 5 show that injection of phospholipid liposomes caused the removal of free cholesterol from HDL and an increase in phospholipid content of the HDL. It has been suggested that the free cholesterol/phospholipid ratio of HDL determines its ability to remove cell cholesterol in vitro (23). According to this view, our observed alterations should increase the ability of HDL to remove tissue cholesterol.

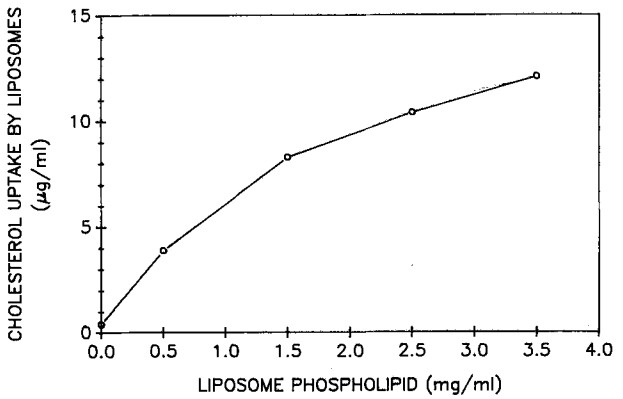

FIG. 8. Cholesterol uptake by liposomes from rabbit red blood cell ghosts. Increasing amounts of egg yolk phospholipid liposomes were incubated with red blood cell ghosts containing 250 μg of phospholipid in 0.15 M NaCl, 0.01 M Tris pH 7.4 for 6 hr at 37°C in a shaking water bath. After incubation membranes were sedimented by centrifugation at 14,000 × *g* for 30 min and cholesterol content in the supernatant was measured.

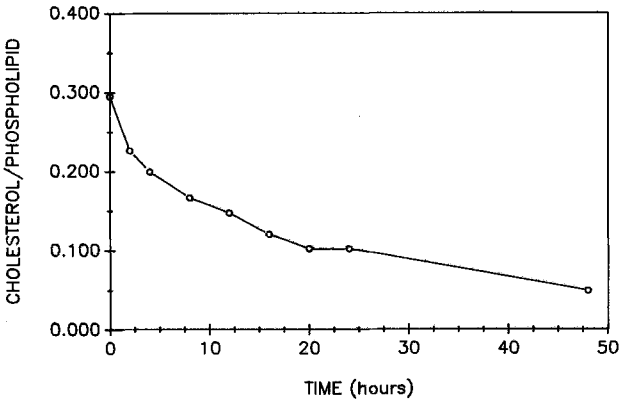

FIG. 9. Time course of cholesterol removal from rabbit red cell ghosts. Red cell ghosts containing 250 μg phospholipids were incubated with 2.5 mg of egg yolk phospholipid liposomes in 1.2 ml of 0.15 M NaCl, 0.01 M Tris, pH 7.4, for various lengths of time at 37°C in a shaking water bath. After incubation, red cell ghosts were sedimented and washed 3 times with buffer at 14,000 × *g*. Cholesterol and phospholipid contents were assayed and the ratio calculated.

TABLE 1

Lack of Change in Red Blood Cells After Injection of Phospholipid Liposomes

	Red blood cell ghosts Cholesterol/phospholipid			Whole blood Hematocrit (%)	
Rabbit	Before	After	% Change	Before	After
1	0.504	0.486	−3.57	38	37
2	0.438	0.423	−3.42	39	38
3	0.462	0.476	+3.03	35	35

Rabbits were injected with 60 mg liposomal phospholipid/kg body weight and blood was collected before and after injection, for measuring hematocrit. Red blood cell ghosts were prepared for measurement of cholesterol and phospholipids.

The fate of cholesterol incorporated into liposomes is not clear. However, many studies have shown that the injected liposomes are preferentially taken up by the liver (24,25). The data in Figure 7 demonstrated that liposomes acquire, in addition to free cholesterol, apolipoproteins E, A-I and traces of C. Because hepatocytes have receptors for apo E and apo A-I (26,27), their presence should target the liposomes for uptake by the liver. Our data support the view that injection of phospholipids has an antiatherogenic effect by enhancing the reverse cholesterol transport mechanism, a hypothesis recently put forth by Williams et al. (28). This enhancement, theoretically at least, may have an antiatherogenic effect.

ACKNOWLEDGMENTS

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REFERENCES

1. Friedman, M., Byers, S.O., and Rosenman, R.H. (1957) *Proc. Soc. Exp. Biol. Med. 95*, 586-588.
2. Byers, S.O., and Friedman, M. (1960) *J. Lipid Res. 1*, 343-348.
3. Altman, R.F.A., de Mendonca, J.M., Schaeffer, G.M.V., Ramos de Souza, J., Baudoli, J.G., da Silva, D.J., and Lopes, C.R.N. (1974) *Arzneim. Forsch. 24*, 11-16.
4. Howard, A.N., Patelski, J., Bowyer, D.E., and Gresham, G.A. (1971) *Atherosclerosis 14*, 17-29.
5. Stafford, W.W., and Day, C.E. (1975) *Artery 1*, 106-114.
6. Williams, K.J. and Scanu, A.M. (1986) *Biochim. Biophys. Acta 875*, 183-194.
7. Williams, K.J., Tall, A.R., Bisgaier, C., and Brocia, R. (1987) *J. Clin. Invest. 79*, 1466-1472.
8. Huang, C.H. (1969) *Biochemistry 8*, 344-351.
9. Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W., and Fu, P.C. (1974) *Clin. Chem. 20*, 470-475.
10. McGowan, M.W., Artiss, J.D., Strandbergh, D.R., and Zak, B. (1983) *Clin. Chem. 29*, 538-542.
11. Zilversmit, D.B., and Davis, A.K. (1950) *J. Lab. Clin. Med. 35*, 155-160.
12. Bartlett, G.R. (1958) *J. Biol. Chem. 234*, 466-468.
13. Terpstra, A.H.M., Woodward, C.J.H., and Sanchez-Muniz, F.J. (1981) *Anal. Biochem. 111*, 149-157.
14. O'Farrel, P.H., Gold, L.M., and Haung, W.M. (1975) *J. Biol. Chem. 250*, 4007-4017.
15. Laemmli, U.K. (1970) *Nature 227*, 680-685.
16. Menzel, H.J., Kladetzky, R.G., and Assman, G. (1983) *Arteriosclerosis 3*, 310-315.
17. Scanu, A.M., and Edelstein, C. (1971) *Anal. Biochem. 44*, 576-588.
18. Dodge, J.T., Mitchell, C., and Hanahan, D.J. (1963) *Arch. Biochem. Biophys. 100*, 119-130.
19. Tall, A.R., Tabas, I., and Williams, K.J. (1986) *Meth. Enzym. 128*, 947-657.
20. Byers, S.O., and Friedman, M. (1957) *Proc. Soc. Exp. Biol. Med. 94*, 452.
21. Bartholow, L.C., and Geyer, R.P. (1982) *J. Biol. Chem. 257*, 3126-3130.
22. Slotte, J.P., and Bjorkerud, S. (1984) *Biochem. J. 220*, 605-608.
23. Arbogast, L.Y., Rothblat, G.A., Leslio, M.H., and Cooper, P.A. (1976) *Proc. Natl. Acad. Sci. USA 73*, 3680-3684.
24. Poste, G., Bucana, C., Raz, A., Bugelski, P., Kirsh, R., and Fidler, I.J. (1982) *Cancer Res. 42*, 1412-1422.
25. Senior, J., Crawley, J.C.W., and Gregoriadas, G. (1985) *Biochim. Biophys. Acta 839*, 1-8.
26. Rifici, V.A., and Eder, H.A. (1984) *J. Biol. Chem. 259*, 13814-13818.
27. Mahley, R.W., Hui, D.Y., Innerarity, T.L., and Weisgraber, K.H. (1981) *J. Clin. Invest. 68*, 1197-1206.
28. Williams, K.J., Werth, V.P., and Wolff, J.A. (1984) *Perspect. Biol. Med. 27*, 417-431.

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Docosahexaenoic Acid and Other Dietary Polyunsaturated Fatty Acids Suppress Leukotriene Synthesis by Mouse Peritoneal Macrophages

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The efficacy of individual ω-3 polyunsaturated fatty acids (PUFA) in altering eicosanoid synthesis in peritoneal macrophages was studied by feeding mice for 10 days a diet containing 2 wt % fat, which included 0.5 wt % ethyl esters of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or linolenic acid (LNA). Upon stimulation with calcium ionophore A23187, macrophages from these animals produced significantly lower amounts of leukotriene C_4, leukotriene B_4 and 12-hydroxyeicosatetraenoic acid, prostaglandin E_2 and 6-keto prostaglandin $F_{1\alpha}$ compared with those obtained from animals on the diets containing olive oil or safflower oil. The decrease in leukotriene synthesis was similar in the animals fed DHA, EPA or LNA diets. This depression of eicosanoids by DHA and EPA was associated with decreased levels of arachidonic acid (AA); however, LA that altered eicosanoids did not have the same effect on AA levels.
Lipids 23, 968-972 (1988).

The role of leukotrienes in inflammatory processes is well documented (1-3). Leukotriene B_4 (LTB_4) is a powerful chemotactic agent, but leukotriene C_4 (LTC_4) and other peptidoleukotrienes enhance vasopermeability and exudation of plasma cells (3-5). Excess or imbalanced production of these leukotrienes may exacerbate pathophysiological states like inflammation, asthma, arthritis and psoriasis (3,4,6,7). Therefore, the regulation of leukotriene production is vital in controlling inflammation and preventing pathophysiological states.

Leukotriene production can be inhibited by agents such as eicosatetraynoic acid, nordihydroguaiaretic acid, BW755C, esculetin or gossypol (3,4,8,9). Certain dietary polyunsaturated fatty acids (PUFA), especially the ω-3 (n-3) PUFA series in fish oils, may modulate leukotriene synthesis and possibly ameliorate inflammatory conditions (10-13). Though fish oils contain both eicosapentaenoic acid (EPA), 20:5n3, and docosahexaenoic acid (DHA) 22:6n3 as the major n-3 PUFA components, the modulating effects of dietary fish oil on eicosanoid synthesis have been generally attributed to EPA, although the effect of DHA is equivocal (13-15). It has been reported that DHA may not affect leukotriene synthesis at the lipoxygenase level in human neutrophils (15) and RBL-1 cell homogenates (12). However, recently we demonstrated that mouse macrophages, enriched with DHA in vitro, produced significantly lower amounts of leukotrienes, compared with the control cells, indicating that DHA can significantly reduce leukotriene synthesis by altering the arachidonic acid (AA) substrate pools utilized for leukotriene synthesis (16).

In order to determine if similar effects can be induced in vivo, mice were fed a diet enriched with DHA. To compare the efficacy of DHA with other unsaturated fatty acids, groups of mice also were fed diets enriched with EPA (20:5n3), linolenic acid (LNA) (18:3n3), olive oil (18:1n9) or safflower oil (18:2n6).

MATERIALS AND METHODS

Materials. Calcium ionophore A23187 (lot 105f-4014), reduced glutathione and cysteine hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO). Ethyl esters of linolenic acid (>99.5% pure) and docosahexaenoic acid (<99.5% pure) and a preparation of eicosapentaenoic acid ethyl esters (53% EPA, 7% docosapentaenoic acid, 13% DHA) were purchased from NuChek Prep (Elysian, MN). Commercial edible olive oil and safflower oil were obtained locally. Leukotriene standards were obtained from Merck Frosst (Montreal, Canada). Radioimmunoassay kits for quantification of prostaglandins (prostaglandin E_2 and 6-keto prostaglandin $F_{1\alpha}$) were purchased from Seragen, Inc. (Boston, MA). Analytical grade solvents were used for the extraction of lipids and eicosanoids.

Animals. Male mice (CD-1 type) weighing 18-20 g were purchased from Charles River (Wilmington, MA) and housed five per cage. A 12 hr light-dark cycle and a temperature of 22°C was maintained in the room.

Diets. Fat-free diet was purchased from ICN Nutritional Biochemicals (Cleveland, OH). All diets were thoroughly mixed with 1.5 wt % olive oil and then supplemented with an additional 0.5 wt % olive oil (Diet A), 0.5 wt % safflower oil (Diet B), 0.5% wt % ethyl ester of LNA (Diet C), 0.5 wt % ethyl ester of EPA (Diet D) and 0.5 wt % DHA (Diet E). The diets were thoroughly mixed after the addition of oils, and small amounts (35 g) were transferred to Whirlpak plastic bags, flushed with nitrogen and stored at 4°C in the dark. The mice received fresh diet every day. There was no oxidation of dietary fats as measured by thiobarbituric acid reactive materials and also by the analysis of fatty acids (17). The fatty acid composition of the diets is shown in Table 1. Mice were fed a fat-free diet for one week to reduce endogenous ω-3 fatty acids and facilitate the rapid uptake of dietary fatty acid esters fed to the animals. The animals on a fat-free diet for one week did not show any symptoms of essential fatty acid deficiency and did not accumulate 20:3n-9 fatty acid. The net production of eicosanoids by macrophage from lab chow-fed animals were: Prostaglandin E_2 (PGE_2) 5.2 ± 0.96 ng/μg DNA and 6 keto Prostaglandin $F_{1\alpha}$ (6 keto $PGF_{1\alpha}$) 32.3 ± 3.16 ng/μg DNA. The corresponding values for macrophages from fat-free-fed animals for one week were PGE_2 5.45 ± 0.9 ng/μg DNA

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HETE, hydroxy fatty acid; LA, linoleic acid; LNA, linolenic acid; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; PGE_2, prostaglandin E_2; $PGF_{1\alpha}$, prostaglandin $F_{1\alpha}$; PUFA, polyunsaturated fatty acid; TXB_2, thromboxane B_2; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; wt %, weight percent(age).

TABLE 1

Fatty Acid Composition of Lipids[a]

Fatty acid	Diet A OO	Diet B SO	Diet C LNA	Diet D EPA	Diet E DHA
14:0	23.33	15.68	21.79	23.39	21.01
16:0	7.54	6.50	4.93	5.69	5.55
16:1	1.07	0.82	0.80	1.09	0.81
18:0	1.31	1.32	0.86	1.25	1.01
18:1	55.91	53.11	43.72	48.66	46.54
18:2n6	9.62	21.86	6.96	7.34	7.23
18:3n3	0.88	0.44	20.46	1.01	0.51
20:4n6	0.00	0.00	0.00	0.31	0.00
20:5n3	0.27	0.20	0.23	8.06	0.93
22:5n6	0.61	0.07	0.10	0.20	0.00
22:5n3	0.00	0.00	0.00	1.10	0.00
22:6n3	0.00	0.00	0.13	1.88	16.42

[a]In diets fed to experimental mice for 10 days (mol percent).

OO, olive oil; SO, safflower oil; LNA, linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

and 6 keto $PGF_{1\alpha}$ 30.1 ± 2.5 ng/μg DNA. Therefore, the fat-free diet for one week did not exert any deleterious effects on macrophages. Following one week on a fat-free diet, the mice were randomized into cages (5 animals per cage) and fed experimental diets for 10 days. Diet A (olive oil diet) was used as the control diet, as in previous studies (18). Diet B was enriched with linoleic acid (LA), 18:2n6; diet C with LNA; diet D with EPA; and diet E was enriched with DHA. The total fat content (2 wt %) was the same in all diets.

Isolation of peritoneal macrophages. After 10 days on the fat-supplemented diet, the mice were sacrificed by ether inhalation. Peritoneal cells were removed in PBS buffer containing 10 IU heparin/ml. Cells were washed and suspended in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (2.5×10^6 cells/ml). Aliquots (2 ml) were dispensed in Falcon tissue culture wells (35 mm) and incubated for 2 hr at 37°C in a humidified chamber containing 5% CO_2. The adhering cells (1.8×10^6 cells) were washed three times in Dulbecco's medium (19). More than 95% of the cells were identified as macrophages by esterase staining and phagocytosis (20). The protein content of the cells in culture, determined by Lowry et al. (21), was 80 ± 10 ng/10^6 cells and the corresponding DNA content, determined with mitramycin (22), was 56 ± 3.2 ng/mg protein (means ± SD; n = 6).

Leukotriene analysis. Macrophages in 1.5 ml Dulbecco's modified Eagle medium were stimulated by incubation with 0.5 μg/ml calcium ionophore A23187, 5 mM cysteine hydrochloride, 1 mM reduced glutathione for 2 hr at 37°C, as previously described (16,23,24). Reactions were terminated by adding 2 ml methanol, and the cells with the medium were transferred to extraction tubes, sonicated for 30 sec at 4°C and centrifuged for 5 min in a clinical centrifuge. The supernatant was removed and the pH was adjusted to pH 3 with 3% formic acid. This solution was adjusted to contain 20% methanol by adding deionized distilled water. These samples were prepared for high performance liquid chromatography (HPLC) by selective extraction of the eicosanoids/leukotrienes by reverse-phase columns (Sep Pak, Waters, Milford, MA), using the methodology described by Borgeat et al. (25). The leukotriene fractions were quantified by HPLC (Waters, Milford, MA) using a Supelco ODS reverse-phase column and a mobile phase of methanol/water (75:25, v/v) in 5 mM ammonium acetate buffer (pH 5.6) containing 1 mM EDTA. Leukotrienes and hydroxy fatty acids (HETE) were identified by retention time compared with authentic standards, by their UV absorption spectra obtained by a diode array spectrophotometric detector and were quantified by their absorbances using extinction coefficients of authentic standards (16).

Prostaglandin analysis. Macrophages were stimulated with calcium ionophore A 23187 (0.5 μg/ml) for 2 hr at 37°C. Prostaglandins were extracted from the medium in three volumes of ethyl acetate and quantified by radioimmunoassay (26). The PGE_2 antiserum had a cross reactivity of less than 1% with TXB_2, 6-keto $PGF_{1\alpha}$, HETE and AA. The 6-keto $PGF_{1\alpha}$ antiserum had a cross reactivity of 0.6% with PGE_2 and less than 0.1% with TXB_2, HETE and AA.

Fatty acid analyses. Because insufficient macrophage material was available for fatty acid analyses, spleen tissue which changes in a manner similar to macrophages in response to fish oil (26–28), were analyzed to monitor changes after feeding experimental diets.

Mice were killed by ether asphyxiation and the spleens were immediately removed and flushed with a buffer, 0.25 M sucrose containing 10 mM Tris and 1 mM EDTA buffer, pH 7.4. After removing the connective tissues, the spleens were weighed, minced using scissors and homogenized at 4°C in 5 vol of 0.05 M Tris HCl buffer, pH 7.4, in a polytron (Kinematics, Lucerne, Switzerland) for 60 sec in 2 × 30 sec bursts at setting 4, followed by a 20 sec burst with an Ultrasonics Model W-10 ultrasonic sonicator (Ultrasonics, Plainview, NY). The homogenate was collected after centrifuging at 600 *g* for 15 min in a Sorval RC5B to remove cell debris and unbroken cells. Lipids were extracted from spleen homogenates by the method of Bligh and Dyer (29). The phospholipids were separated from neutral lipids on HPTLC plates using chloroform/methanol (80:20, v/v). The lipids were saponified with 0.5 N KOH in methanol and the fatty acids were methylated with diazomethane. The fatty acid methyl esters were separated using a Hewlett-Packard 5880A gas chromatograph (Hewlett-Packard, Avondale, PA) on a 0.75 mm by 60 MSP2330 capillary column (Supelco, Inc., Bellefonte, PA) with hydrogen as carrier gas. Fatty acids were identified by comparing retention times with fatty acid methyl ester standards and quantified using pentadecanoic acid as internal standard (30).

Statistical methods. Results were statistically evaluated by Statistical Analysis System using the General Linear Models procedure and the least significant difference mean-separation determination (SAS Institute, Chapel Hill, NC) (31).

RESULTS AND DISCUSSION

The amounts of diet consumed (ca. 5 g/day/mouse) and the weight gains (6.1 ± 0.3 g/mouse in 10 days, mean ± SD, n = 10 per group) by the animals were similar in all dietary groups.

TABLE 2

Fatty Acid Composition of Phospholipids from Spleens[a]

Fatty acid	Diet A OO	Diet B SO	Diet C LNA	Diet D EPA	Diet E DHA
14:0	ND	ND	ND	ND	ND
16:0	35.38 ± 2.86	37.05 ± 0.15	32.64 ± 1.55	34.02 ± 1.81	40.44 ± 1.37
16:1	2.27 ± 0.21	2.26 ± 0.02	1.98 ± 0.05	2.23 ± 0.21	2.46 ± 0.07
18:0	13.73 ± 0.53^{b,c}	13.80 ± 0.12^{b,c}	14.52 ± 0.18^{b}	13.66 ± 0.68^{b,c}	12.28 ± 0.40^{c}
18:1n9	21.54 ± 1.25	22.14 ± 0.07	20.00 ± 0.33	17.27 ± 2.35	20.86 ± 0.40
18:2n6	2.94 ± 0.19^{b}	4.47 ± 0.08^{c}	2.46 ± 0.05^{d}	2.65 ± 0.20^{b,d}	2.67 ± 0.07^{b,d}
18:3n3	1.11 ± 0.13	0.90 ± 0.01	0.87 ± 0.09	1.00 ± 0.34	0.67 ± 0.06
20:3n6	1.62 ± 0.15	1.83 ± 0.03	1.16 ± 0.04	1.55 ± 0.64	0.74 ± 0.06
20:4n6	15.03 ± 1.18^{b}	13.37 ± 0.23^{b}	13.30 ± 0.48^{b}	10.75 ± 0.32^{c}	8.04 ± 0.38^{d}
20:5n3	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	1.82 ± 0.11^{c}	3.68 ± 0.13^{d}	1.21 ± 0.15^{e}
22:4n6	0.91 ± 0.05^{b}	0.45 ± 0.01^{c}	0.30 ± 0.05^{c,d}	0.55 ± 0.28^{b,c}	0.00 ± 0.00^{c,d}
22:4n3	1.76 ± 0.25^{b}	1.36 ± 0.04^{b,c}	1.31 ± 0.10^{b,c}	1.13 ± 0.26^{c}	0.46 ± 0.03^{d}
22:5n6	0.06 ± 0.15^{b}	0.77 ± 0.05^{b,c}	0.52 ± 0.06^{c,d}	0.45 ± 0.12^{d}	0.49 ± 0.05^{c,d}
22:5n3	0.33 ± 0.06^{b,c}	0.15 ± 0.00^{b}	2.93 ± 0.35^{d}	4.45 ± 0.37^{e}	0.94 ± 0.03^{c}
22:6n3	2.42 ± 0.37^{b}	1.43 ± 0.04^{b}	6.21 ± 0.69^{c}	6.61 ± 0.64^{c}	8.73 ± 0.71^{d}

[a]Isolated from mice maintained for 10 days on diets containing different unsaturated fatty acids (mol %).
^{b,c,d,e}Means followed by different letters in the same row are significantly different ($p < 0.05$).
ND, not detected. Mean ± SEM, n = 3.

Fatty acid composition. Spleen is an important site for monocyte/macrophage production and it responds to dietary fatty acids in a way similar to those observed with macrophages (26–28,32). Therefore, the changes induced in spleen phospholipid fatty acid composition by the different dietary fatty acids were determined. The animals on safflower oil diets accumulated significant amounts of LA compared to those on olive oil diets (Table 2). The AA levels in spleens of safflower oil-fed animals, however, were similar to those on olive oil diets, despite the fact that safflower oil diets contained threefold more LA, the precursor of AA (Table 1), indicating that the metabolism of LA to AA is highly regulated. Animals on LNA-enriched diets (Diet C) contained similar amounts of AA, but had significant amounts of 22:5n3 (DPA) and DHA, the elongated and desaturated products of LNA. Significantly, LNA did not accumulate in the spleen phospholipids of animals on LNA-enriched diets. The animals consuming EPA-enriched diets (Diet D) accumulated mostly DPA and DHA, the elongation and desaturation products of EPA. The accumulation of these fatty acids was associated with the displacement of AA from the phospholipids. The animals on the DHA-enriched diet (Diet E) contained significantly higher amounts of DHA in spleen phospholipids, which was accompanied by a decrease of AA (Table 2). A small accumulation of DPA and EPA, the retroconversion product of DHA, was observed in spleen phospholipids of animals on the DHA-enriched diets. These data indicate that the metabolism of PUFA are differentially regulated. While LA (from the safflower oil diet) accumulated in the spleen lipids without any further metabolism, LNA was not accumulated as such but as its elongated and desaturated products. However, EPA accumulated as the parent fatty acid and also as its products, DPA and DHA. A small amount of these fatty acids may have been obtained directly from the diets since EPA-enriched diets also contained small amounts of DPA and DHA (Table 1). However, our studies of the modifications of macrophage in vitro with pure EPA have shown that significant amounts of EPA were elongated to DPA, but not desaturated to DHA by macrophages (16). Similar results have been observed in human polymorphonuclear leukocytes (33,34). Docosahexaenoic acid accumulated mostly as DHA in spleen phospholipids, with only a small amount being retroconverted to DPA and EPA. These results are consistent with those observed in humans, rat liver and endothelial cells (35–37). While LA (from safflower oil) and LNA did not affect AA levels, EPA and DHA displaced AA from spleen phospholipids.

Leukotriene and 12-HETE synthesis. Macrophages are major sources of eicosanoids (38,39) and prostaglandin and leukotriene synthesis is sensitive to the influence of dietary fats (11,26). Therefore, macrophages were analyzed to ascertain the efficacy of different PUFA in modulating eicosanoid production. The resident peritoneal macrophages synthesized negligible amounts of leukotrienes in the absence of a stimulus (23,40,41). Following exposure to calcium ionophore (A23187), the macrophages synthesized significant amounts of LTC_4 and LTB_4 from endogenous substrates (16,23). Upon stimulation, the net production of LTC_4, LTB_4 and 12-HETE, in macrophages isolated from animals on diets enriched in n-3 PUFA, was significantly lowered ($p < 0.05$), compared with the macrophages isolated from animals on safflower or olive oil diets (Fig. 1). These results indicated that n-3 PUFA significantly lowered the net production of leukotrienes in macrophages. The magnitude of the decreases in leukotrienes observed from animals in LNA, EPA or DHA diets was comparable indicating that, irrespective of the chain length and degree of unsaturation, the n-3 PUFA in the diet lowered leukotriene production in macrophages. Significantly, DHA was very effective in reducing leukotrienes. Unlike that in rats (11), the mouse

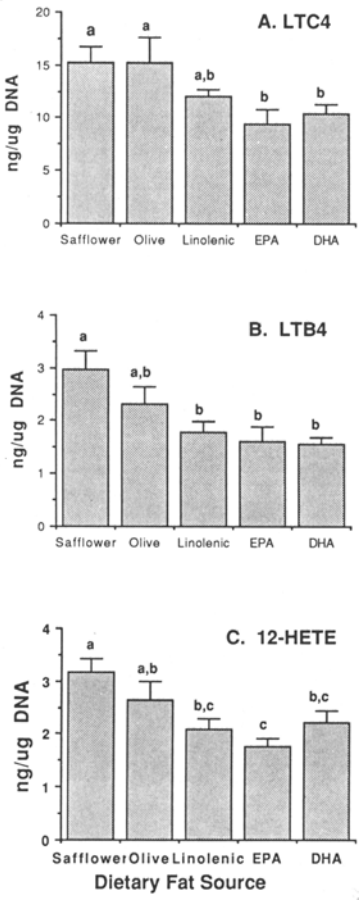

FIG. 1. Histograms showing the relative efficacies of different dietary unsaturated fatty acids in depressing the net production of A) LTC_4, B) LTB_4 and C) 12-HETE by mouse peritoneal macrophages. Macrophages isolated from mice on different dietary fats were stimulated with calcium ionophore A23187 (0.5 μg/ml) for 2 hr at 37°C. The leukotrienes were extracted and analyzed by HPLC as described in methods. Results are mean ± SEM from 5 animals. Means with different letters above the histograms are significantly different ($p < 0.05$) than each other. Histograms with same notation are not different than each other.

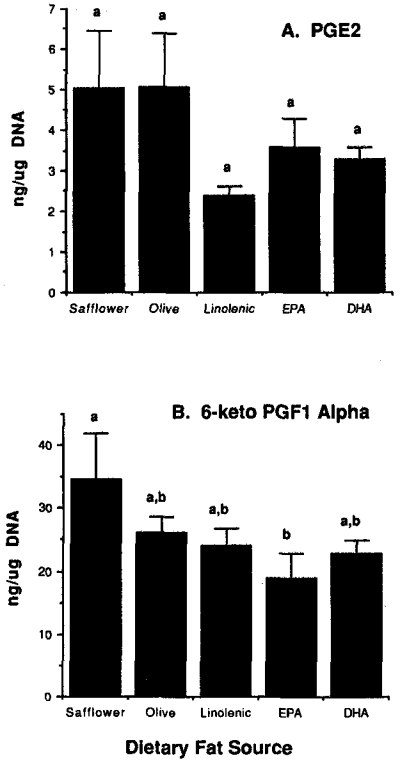

FIG. 2. The effects of different dietary unsaturated acids on the net production of A) PGE_2 and B) 6-keto $PGF_{1\alpha}$ by mouse peritoneal macrophages. Macrophages isolated from mice on different dietary fats were stimulated with calcium ionophore A23187 (0.5 μg/ml) for 2 hr at 37°C. The prostaglandins were extracted in three volumes of ethyl acetate and quantified by radioimmunoassay as described in methods. Results are mean ± SEM from 5 animals. Histograms with different letters are significantly different than each other ($p < 0.05$).

macrophage did not produce 5-series leukotrienes in EPA-enriched cells.

Unlike EPA and DHA, LNA did not displace AA from membrane lipids but, as its elongated products (DPA and DHA), it was still effective in suppressing leukotriene synthesis in macrophages. Therefore, the displacement of AA from tissue pools is not essential, but the incorporation of n-3 PUFA is apparently necessary for suppressing leukotriene production in macrophages. The spleens from animals exposed to EPA-enriched diets contained 50% less n-3 PUFA (ca. 8 mol %) compared with DHA (ca. 16 mol %) or LNA (ca. 18 mol %) diets (Table 1), but caused a similar decrease in leukotriene production. Thus, n-3 PUFA, even as low as 8 mol % in the diet, can suppress leukotriene synthesis. Further studies, to evaluate the minimum amount of n-3 PUFA in the diet needed to suppress leukotriene synthesis in macrophages and to identify the possible role of DPA in this reaction, are in progress.

This study demonstrates that DHA indeed suppresses leukotriene synthesis in macrophages, which agrees with our earlier studies on macrophages modified in vitro with purified DHA (16). Earlier studies with human neutrophils (15) and RBL-1 cell homogenates (12) indicated that DHA is ineffective in modulating leukotriene synthesis in short-term incubations. However, our dietary studies and in vitro studies demonstrated that DHA is as effective as other n-3 PUFA in suppressing leukotriene synthesis, provided a longer time of equilibration with DHA was allowed.

Prostaglandin synthesis. In addition to leukotrienes, macrophages also synthesize prostaglandins upon stimulation with calcium ionophore (38). Macrophages from animals on n-3 PUFA diets produced lower amounts of PGE_2 and 6-keto $PGF_{1\alpha}$ compared with those animals eating a diet containing safflower oil or olive oil (Fig. 2). This is in agreement with our previous results, where n-3 PUFA from a menhaden oil diet or enrichment of macrophages with pure n-3 PUFA lowered prostaglandin synthesis (26,42).

In conclusion by dietary and in vitro modifications, we have demonstrated that DHA, after equilibrating in the cellular lipids, is capable of suppressing leukotriene synthesis in macrophages and its effect is as significant as those observed with EPA. Therefore, in addition to EPA, one should also consider the DHA component of fish oils when evaluating their efficacies in modulating eicosanoid-mediated functions, including inflammatory reactions, cardiovascular functions and perhaps cancer (43–45).

ACKNOWLEDGMENTS

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REFERENCES

1. Lewis, R.A., and Austen, K.F. (1984) *J. Clin. Invest. 73,* 889-897.
2. Marcus, A.J. (1984) *J. Lipid Res. 25,* 1511-1516.
3. Samuelsson, B. (1983) *Science 220,* 568-575.
4. Stjernschantz, J. (1984) *Med. Biol. 62,* 215-230.
5. Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, E., and Smith, M.J.H. (1980) *Nature 286,* 64-65.
6. Ford-Hutchinson, A.W. (1985) in *Biochemistry of Arachidonic Acid Metabolism* (Lands, W.E.M., ed.) pp. 269-285, Martinus-Nijhoff, Boston, MA.
7. Leslie, C.A., Gonnerman, W.A., Ullman, D.M., Hayes, K.C., Franzblau, C., and Cathcart, E.S. (1985) *J. Exp. Med. 162,* 1336-1349.
8. Higgs, G.A., and Vane, J.R. (1983) *Br. Med. Bull. 3,* 265-270.
9. Hamasaki, Y., and Tai, H.H. (1985) *Biochim. Biophys. Acta 834,* 37-41.
10. Robinson, D.R., Prickett, J.D., Polisson, R., Steinberg, A.D., and Levine, L. (1985) *Prostaglandins 30,* 51-63.
11. Leitch, A.G., Lee, T.H., Ringel, E.W., Prickett, J.D., Robinson, D.R., Pyre, S.G., Corey, E.J., Drazen, J.M., Austen, K.F., and Lewis, R.A. (1984) *J. Immunol. 132,* 2559-2565.
12. Corey, E.J., Shih, C., and Cashman, J.R. (1983) *Proc. Natl. Acad. Sci. USA 80,* 3581-3584.
13. Lee, T.H., Hoover, R.L., Williams, D., Sperling, R.I., Ravalese, J., Spur, W.W., Robinson, D.R., Corey, E.J., Lewis, R.A., and Austen, K.F. (1985) *N. Engl. J. Med. 312,* 1217-1224.
14. Prescott, S.M. (1984) *J. Biol. Chem. 259,* 7615-7620.
15. Lee, T.H., Mencia-Huerta, J.M., Shih, C., Corey, E.J., Lewis, R.A., and Austen, K.F. (1984) *J. Clin. Invest. 74,* 1922-1933.
16. Lokesh, B.R., German, J.B., and Kinsella, J.E. (1988) *Biochim. Biophys. Acta 958,* 99-107.
17. Lokesh, B.R., Mathur, S.N., and Spector, A.A. (1981) *J. Lipid Res. 22,* 905-915.
18. German, J.B., Lokesh, B.R., and Kinsella, J.E. (1987) *Prostaglandins Leukotrienes Med. 30,* 69-76.
19. Lokesh, B.R., and Kinsella, J.E. (1985) *Biochim. Biophys. Acta 845,* 101-108.
20. Miller, G.A. and Monahan, P.S. (1981) in *Methods for Studying Mononuclear Phagocytes* (Adams, D.O., Edelson, P.J., and Koren, H., eds.) pp. 367-372, Academic Press, New York, NY.
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
22. Hill, B.T., and Whatley, S. (1975) *FEBS Lett. 56,* 20-23.
23. Abe, M., Kawazoe, Y., Tsunematsu, H., and Sigematsu, N. (1985) *Biochem. Biophys. Res. Commun. 127,* 15-23.
24. Tripp, C.X., Mahoney, M., and Needleman, P. (1985) *J. Biol. Chem. 260,* 5895-5898.
25. Borgeat, P., Fruteau de Laclos, B., Rabinovitch, H., Picard, S., Braquet, P., Herbert, J., and Laviolette, M.L. (1984) *J. Allergy Clin. Immunol. 74,* 310-317.
26. Lokesh, B.R., Hsieh, H.L., and Kinsella, J.E. (1986) *J. Nutr. 116,* 2547-2552.
27. Lokesh, B.R., Hsieh, H.L., and Kinsella, J.E. (1986) *Ann. Nutr. Metab. 30,* 357-364.
28. Zuniga, M.E., Lokesh, B.R., and Kinsella, J.E. (1987) *Nutr. Res. 7,* 299-306.
29. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
30. Swanson, J.E., Black, M.M., and Kinsella, J.E. (1987) *J. Nutr. 117,* 824-832.
31. Goodright, J.H. (1979) Analysis of Variance In: *SAS User's Guide* (Helmig, J.T. and Council, K.A., eds.) pp. 113-229, SAS (Statistical Analysis System) Institute, Raleigh, NC.
32. Tischendorf, F. (1985) *Experientia 41,* 145-152.
33. Terano, T., Salmon, J.A., and Moncada, S. (1984) *Biochem. Pharmacol. 33,* 3071-3076.
34. Terano, T., Hirai, A., Tamura, Y., Kumagai, A., Yoshida, S. (1987) *Adv. Prostaglandin Thromboxane Leukotriene Res. 17B,* 880-885.
35. Fischer, S., Vischer, A., Mursic, V.P., and Weber, P.C. (1987) *Prostaglandins 34,* 367-375.
36. Schlenk, H., Sand, D.M., and Gellerman, J.L. (1969) *Biochim. Biophys. Acta 187,* 201-207.
37. Hadjagapiou, C. and Spector, A.A. (1987) *Arch. Biochem. Biophys. 253,* 1-9.
38. Humes, J.L., Sandowski, S., Galavage, M., Goldenberg, M., Subers, E., Bonney, R.J., and Kuehl, F.A., Jr. (1982) *J. Biol. Chem. 257,* 1591-1594.
39. Stenson, W.F., and Parker, C.W. (1980) *J. Immunol. 125,* 15-18.
40. Humes, J.L., Opas, E.E., Galavage, M., Soderman, D., and Bonney, R.J. (1986) *Biochem. J. 233,* 199-206.
41. Rouzer, C.A., Scott, W.A., Hamill, A.L., and Cohn, Z.A. (1980) *J. Exp. Med. 152,* 1236-1247.
42. Lokesh, B.R. and Kinsella, J.E. (1987) *Immunobiology 175,* 406-419.
43. Bhattacherjee, P., and Eakins, K.E. (1984) in *The Leukotrienes-Chemistry and Biology* (Chakrin, L.W. and Bailey, D.M., eds.) pp. 195-214, Academic Press, Inc., Orlando, FL.
44. Herold, P.M., and Kinsella, J.E. (1986) *Am. J. Clin. Nutr. 43,* 566-598.
45. Karmali, R.A. (1987) *Am. J. Clin. Nutr. 45,* 225-229.

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Dietary Linoleic Acid and the Fatty Acid Profiles in Rats Fed Partially Hydrogenated Marine Oils

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The influence of the linoleic acid levels of diets containing partially hydrogenated marine oils (HMO) rich in isomeric 16:1, 18:1, 20:1 and 22:1 fatty acids on the fatty acid profiles of lipids from rat liver, heart and adipose tissue was examined. Five groups of rats were fed diets containing 20 wt % fat—16% HMO + 4% vegetable oils. In these diets, the linoleic acid contents varied between 1.9% and 14.5% of the dietary fatty acids, whereas the contents of *trans* fatty acids were 33% in all groups. A sixth group was fed a partially hydrogenated soybean oil (HSOY) diet containing 8% linoleic acid plus 32% *trans* fatty acids, mainly 18:1, and a seventh group, 20% palm oil (PALM), with 10% linoleic acid and no *trans* fatty acids.

As the level of linoleic acid in the HMO diets increased from 1.9% to 8.2%, the contents of (n-6) polyunsaturated fatty acids (PUFA) in the phospholipids increased correspondingly. At this dietary level of linoleic acid, a plateau in (n-6) PUFA was reached that was not affected by further increase in dietary 18:2 (n-6) up to 14.5%. Compared with the HSOY- or PALM-fed rats, the plateau values of 20:4(n-6) were considerably lower and the contents of 18:2(n-6) higher in liver phosphatidylcholines (PC) and heart PC. Heart phosphatidylethanolamines (PE) on the contrary, had elevated contents of 20:4(n-6), but decreased 22:5(n-6) compared with the PALM group.

All groups fed HMO had similar contents of *trans* fatty acids, mainly 16:1 and 18:1, in their phospholipids, irrespective of the dietary 18:2 levels, and these contents were lower than in the HSOY group.

High levels of linoleic acid consistently found in triglycerides of liver, heart and adipose tissue of rats fed HMO indicated that feeding HMO resulted in a reduction of the conversion of linoleic acid into long chain PUFA that could not be overcome by increasing the dietary level of linoleic acid.
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Partially hydrogenated fats used for the manufacturing of margarines and other fats for human consumption are made from vegetable oils in the U.S. (1), but in Europe may include considerable quantities of marine oils (2), particularly in low linoleic acid margarines. The isomeric octadecenoic acids present in partially hydrogenated vegetable oils are incorporated into various tissues of the rat (3–5), as are isomers from partially hydrogenated marine oils (HMO) (6,7). Post mortem studies have also demonstrated presence of isomeric *cis* and *trans* octadecenoates (8,9) in humans.

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Abbreviations: EFA, essential fatty acid; HMO, partially hydrogenated marine oil; HSOY, partially hydrogenated soybean oil; OO, olive oil; PALM, palm oil; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; SO, sunflowerseed oil; TG, triglyceride; VLDL, very low density lipoprotein; GLC, gas liquid chromatography; SEM, standard error of the mean; TLC, thin layer chromatography.

It has been shown, using liver microsomes from rats raised on essential fatty acid (EFA)-deficient diets, that isomeric octadecenoic acids may competitively inhibit the Δ6-desaturation of 18:2(n-6) into 18:3(n-6) and the Δ5-desaturation of 20:3(n-6) into 20:4(n-6) (10,11). Feeding trials using *trans,trans* 18:2 have also demonstrated a decreased formation of arachidonic acid (12,13).

Partially hydrogenated oils of marine origin differ from those of vegetable origin in their significant contents of 20:1- and 22:1-*cis* and *trans* fatty acids.

Only a few studies on the effects of HMO in EFA-sufficient diets have been published so far. In these studies, a diet with a high level of HMO and a low level of linoleic acid was compared with a reference diet containing a vegetable oil with a much higher linoleic acid level. Using such an experimental design, it was shown that rats fed an HMO + 18:2 diet had reduced levels of 20:4(n-6), but increased levels of 18:2(n-6) and 20:3(n-6), but rats fed a partially hydrogenated peanut oil diet, with similar contents of *trans* fatty acids and linoleic acid as the former, had a smaller decrease in 20:4(n-6) and only accumulated 18:2(n-6) (14,15). This could be explained by reduced Δ6- and Δ5-desaturase activities following intake of HMO, but rats fred partially hydrogenated peanut oil had only reduced Δ6-desaturation (16,17). Also, in accordance with former reports, this study demonstrated that partially hydrogenated fats aggravated the state of EFA deficiency, HMO being more potent in this respect than partially hydrogenated peanut oil (18).

In the present experiment, we used diets with various levels of linoleic acid ranging from 1.9% to 14.5% of the dietary fat, with the dietary level of HMO maintained constant, providing 33% *trans* fatty acids. This design allowed us to study if it was possible to overcome the inhibitory effects of HMO on the conversion of linoleic acid simply by increasing the dietary level of linoleic acid eventually to a level higher than that of the reference group not fed HMO. The effects of chain lengths of isomeric fatty acids were examined in a group fed a diet containing partially hydrogenated soybean oil (HSOY) with an intermediate level of linoleic acid. The fatty acid profiles of major phospholipids and triglycerides (TG) were examined in liver, heart and adipose tissue.

MATERIALS AND METHODS

Animal experiments. Eighty-four weanling male Wistar rats (specific-pathogen-free, Møllegaard Laboratory, Ll. Skensved, Denmark) were divided into seven groups, each with 12 rats of similar average weights. The rats received a diet of the following composition (wt %): casein (Holstebro Maelkekondenserings-fabrik), 20%; fat, 20%; sucrose, 10%; maize-starch (Maizena-Compagniet A/S, Copenhagen), 40%; cellulose powder, MN 100 (Machery Nagel, GFR), 4%; vitamin mixture, 0.5%; choline chloride, 0.5%; and salt mixture (including trace elements), 5.0%. The compositions of the vitamin mixture and the trace elements were as previously described (18). The dietary

fats were partially hydrogenated marine oil (HMO) ("Sandarit," Jahres Fabrikker A/S, Sandefjord, Norway); olive oil (OO) (Pharmacopea Danica 48, Mecobenzon A/S, Copenhagen, Denmark); sunflowerseed oil (SO) (Aarhus Oliefabrik A/S, Aarhus, Denmark); partially hydrogenated soybean oil (HSOY) (Solofabrikken A/S, Sønderborg, Denmark); and palm oil (PALM) (Solofabrikken A/S, Sønderborg, Denmark). The dietary fats were mixed into seven different diets (Table 1).

The rats were caged in pairs at 25°C and a relative humidity of 45%. Diets and water were supplied ad libitum. The rats were examined and weighed weekly. After 10 weeks, the rats were decapitated and liver, heart and a sample of adipose tissue were immediately excised.

Analyses of lipids. Total lipids were extracted from liver or heart, according to Folch et al. (19), and determined gravimetrically. The total phospholipid content was quantitated as inorganic phosphorous (20). Adipose tissue was extracted with chloroform.

Phospholipids were separated by thin layer chromatography (TLC) (21), saponified with 0.5 N NaOH in methanol, followed by methylation of fatty acids with 20% BF_3 in methanol containing 0.02% hydroquinone. Methyl esters were analyzed by gas liquid chromatography (GLC) on a Hewlett-Packard 5830A instrument with FID detector using a 9 ft × 2 mm i.d. silanized glass column packed with GP 10% SP-2330 on Chromosorb W/AW, Supelco Inc., Bellefonte, PA. Initial oven temperature was 190°C for 1 min, followed by temperature programming at 1°C/min to 235°C. Injection was on-column. Carrier gas was helium at an initial flow rate of 16 ml/min. The distributions between *cis* and *trans* isomers were determined by GLC on a 20 ft × 1/8 in. o.d. stainless steel column packed with 15% OV-275 on 100/120 Chromosorb P AW-DMCS, Supelco Inc., at 220°C and a helium flow of 10 ml/min (2).

Triglycerides (TG) of total lipid extracts were prepared by TLC on 0.5 mm Silica Gel G plates using hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as solvent. The triglycerides were methylated, after addition of 15:0 as internal standard for quantitation, and analyzed by GLC as described above.

TABLE 1

Fatty Acid Composition of Dietary Fats (calculated from individual fats)[a]

	Dietary fat						
Fatty acid	Group 1 16% HMO + 4% OO (%)	Group 2 16% HMO + 3% OO + 1% SO (%)	Group 3 16% HMO + 2% OO + 2% SO (%)	Group 4 16% HMO + 1% OO + 3% SO (%)	Group 5 16% HMO + 4% SO (%)	Group 6 10% HSOY + 9% OO + 1% SO (%)	Group 7 20% PALM (%)
12:0	0.1	0.1	0.1	0.1	0.1	—	—
14:0	6.5	6.5	6.5	6.5	6.5	0.1	—
14:1 *t*	0.1	0.1	0.1	0.1	0.1	—	—
14:1 *c*	0.3	0.3	0.3	0.3	0.3	—	—
14:2 *c,t/t,c*	0.4	0.4	0.4	0.4	0.4	—	—
16:0	15.2	14.8	14.5	14.2	13.9	12.1	46.1
16:1 *t*	5.2	5.2	5.2	5.2	5.2	0.1	—
16:1 *c*	3.1	3.0	2.9	2.9	2.8	0.6	—
16:2 *c,t/t,c*	0.4	0.4	0.4	0.4	0.4	—	—
18:0	4.1	4.2	4.3	4.5	4.6	3.9	5.7
18:1 *t*	7.4	7.4	7.4	7.4	7.4	26.6	—
18:1 *c*	19.7	16.8	13.9	11.0	8.2	42.7	36.8
18:2 *c,t/t,c*	2.2	2.2	2.2	2.2	2.2	4.7	—
18:2 *c,c*	1.9	5.1	8.2	11.3	14.5	8.0	10.1
18:3 *c,t/t,c*	0.1	0.1	0.1	0.1	0.1	0.8	—
18:3 *c,c,c*	0.1	0.1	0.1	0.1	0.1	0.4	0.2
20:0	2.4	2.4	2.4	2.4	2.4	—	—
20:1 *t*	6.6	6.6	6.6	6.6	6.6	—	—
20:1 *c*	5.9	5.9	5.9	5.9	5.9	—	—
20:2 *c,t/t,c*	2.9	2.9	2.9	2.9	2.9	—	—
22:0	1.8	1.8	1.8	1.8	1.8	—	—
22:1 *t*	6.6	6.6	6.6	6.6	6.6	—	—
22:1 *c*	5.1	5.1	5.1	5.1	5.1	—	—
22:2 *c,t/t,c*	1.5	1.5	1.5	1.5	1.5	—	—
Σ *trans* fatty acids	33	33	33	33	33	32	—
18:2 *c,c*	1.9	5.1	8.2	11.3	14.5	8.0	10.1
Ratio % 18:2/% *trans*	0.06	0.15	0.25	0.34	0.44	0.25	—

[a]Weight percentages determined by GLC.

Determination of catalase activity. As an estimate of the peroxisomal β-oxidation capacity, the contents of peroxisomes in liver fractions were determined by the activity of catalase. The decrease in hydrogen peroxide absorption at 240 nm was monitored as previously described (14).

Statistics. Results were expressed as the mean and the standard error of the mean (SEM). Differences in contents of the major (n-6) fatty acids between the HMO groups, the HSOY group and the PALM group were established by the Newman-Keuls range test (22).

RESULTS

Dietary fats. Partially hydrogenated marine oil (HMO), olive oil (OO) and sunflowerseed oil (SO) were combined (Table 1) to give five dietary fats, groups 1–5, with similar levels of *trans* fatty acids, 33%. The *trans* fatty acids primarily consisted of monoenes as well as small contents of *cis,trans* dienes with chain lengths of C14 to C22. *Trans,trans* dienes were absent. The linoleic acid levels varied from 1.9% to 14.5% of the dietary fats. Group 6 was fed an HSOY diet providing 8% linoleic acid and 32% *trans* acids. Group 7 was a reference group fed unhydrogenated palm oil containing 10% linoleic acid and no isomeric fatty acids. The combinations of dietary fats used in the present experiment allowed for the following comparisons: (1) effect of dietary level of linoleic acid on the formation of polyunsaturated fatty acids (PUFA), groups 1–5; (2) effects of HMO vs HSOY at one dietary level of linoleic acid, group 3 vs group 6; (3) effects of partially hydrogenated fats vs unhydrogenated fats at comparable levels of linoleic acid, groups 3, 6 and 7.

Animal experiments. No significant differences in growth or general performance were detected. The food intakes were similar in all groups.

Liver. In liver phosphatidylcholines (PC) (Table 2), the contents of 20:4(n-6) and 22:5(n-6) were consistently lower in the rats fed HMO, groups 1–5, than in the rats fed PALM. In groups 1–3, this could be explained by a limited dietary supply of (n-6) fatty acids, because in these groups increases in the dietary linoleic acid from 1.9% to 8% were paralleled by increased formation of PUFA. However, a plateau in the contents of (n-6) PUFA was reached at a dietary level of linoleic acid of about 8% and increases in dietary linoleic acid to levels even considerably above that of the reference group could not further elevate the levels of PUFA in liver PC of rats fed HMO. In the rats fed HMO, group 1–5, increased contents of 18:2(n-6) and 20:3(n-6) compared to the PALM group could indicate effects of the diet on the desaturase enzymes. The HSOY diet (group 6) apparently affected the PUFA formation less than did the comparable HMO diet (group 3) and also caused less accumulation of 18:2(n-6), but compared to the PALM diet, an effect on the 20:4(n-6) was still evident in PC.

In the phosphatidylethanolamines (PE) (Table 2), the levels of 20:4(n-6) in groups 1–5 were much less influenced by the dietary contents of linoleic acid than observed for PC. At a dietary content of 8% linoleic acid, a level of 20:4(n-6) similar to that of the PALM group was reached, and further increases were not observed. However, the 22:5(n-6) contents of group 1–5 were significantly lower and the contents of 18:2(n-6) were higher than in the PALM group, still indicating a reduced conversion of linoleic acid in the HMO groups.

Trans fatty acids deposited in HMO fed groups were solely 16:1, 18:1 and 18:2 *cis,trans,* but *trans*-20:1 and 22:1 despite their abundances in the diets, were only detected as traces in PC or PE, indicating that chain shortening of these monoenes had taken place. As the dietary levels of 18:2(n-6) were augmented, the incorporation of *trans* 18:1 also increased, whereas the levels of saturated fatty acids decreased. The total depositions of *trans* fatty acids were considerably higher in the HSOY group, group 6, than in the HMO groups, although the dietary levels were similar.

The TG of liver (Table 3) reflected increasing contents of 18:2(n-6) in the diets, groups 1–5, but the depositions far exceeded the levels in the groups fed HSOY or PALM. In the HMO groups 1–5, the total *trans* fatty acid contents were lower, but the *trans* 16:1 level higher than in the HSOY group.

Heart. In heart PC (Table 4) of rats fed HMO, groups 1–5, the contents of 20:4(n-6) reached a plateau at a dietary level of 18:2(n-6) of 8%, and as in liver PC, further increases in dietary 18:2 did not result in increased formation of 20:4(n-6), which was lower than in the PALM group. Depositions of 18:2(n-6) indicated reduced conversion of linoleic acid.

In PE (Table 4), the contents of 20:4(n-6) seemed little influenced by the dietary 18:2(n-6) levels in HMO-fed rats. The levels of 20:4(n-6), however, were considerably higher in the HMO-fed rats, groups 1–5, than in the HSOY-fed, group 6, or PALM-fed, group 7. Compared to the PALM group, all the HMO- and the HSOY-fed rats had very low contents of 22:5(n-6), pointing to reduced conversion of arachidonic acid when isomeric fatty acids were fed. Also, in PE, the *trans* fatty acid contents were smaller, following intake of HMO, compared with HSOY.

In the HMO-fed rats, groups 1–5, changes in dietary 18:2(n-6) levels were reflected in the heart TG (Table 3), leading to a higher deposition of 18:2 in groups 4 and 5 than in the PALM group.

Adipose tissue. In the HMO-fed rats, more 18:2 was deposited in the adipose tissue (Table 3) than in the HSOY- or PALM-fed rats at similar dietary levels of 18:2. The *trans* fatty acids deposited were *trans*-16:1 and *trans*-18:1 in the HMO-fed groups, but primarily *trans*-18:1 was found in the HSOY group.

Catalase. The rats fed HMO diets had higher catalase activities in liver (Table 5) than rats fed HSOY or PALM diets, indicating an increased peroxisomal activity in accordance with the low levels of liver TG observed in the HMO-fed rats (Table 3).

DISCUSSION

In the formation of long chain PUFA from dietary linoleic acid, the initial Δ6-desaturation of linoleic acid into γ-linolenic acid is rate-limiting (23,24). It has been demonstrated that both the Δ6-desaturation and the subsequent Δ5-desaturation of 20:3(n-6) into 20:4(n-6) can be affected by isomeric fatty acids present in partially hydrogenated fats. This has been shown by Mahfouz et al. (10,11), who found competitive effects of individual *cis* and *trans* octadecenoic acids using microsomes from rats reared on EFA-deficient diets devoid of isomeric fatty acids. Other

investigators (16,17) used microsomes from rats raised on EFA-sufficient diets containing high levels of HMO or partially hydrogenated peanut oil and found reduced conversion of 20:3(n-6) in the HMO group. In these experiments, the microsomal pool of free fatty acids that could act as competitive inhibitors was insignificant. It was thus concluded that the changes in fatty acid profiles of membrane lipids resulting from the long-term dietary treatment could affect the desaturase activities.

Apparently, two effects of isomeric fatty acids may be operative: A direct competitive inhibition of the desaturase by isomeric fatty acids and a membrane effect due

TABLE 2

Fatty Acids in Phosphatidylcholines and Phosphatidylethanolamines and Total Phospholipid Content in Rat Liver[a]

	Dietary fat						
Fatty acid	Group 1 16% HMO[b] + 4% OO (%)	Group 2 16% HMO + 3% OO + 1% SO (%)	Group 3 16% HMO + 2% OO + 2% SO (%)	Group 4 16% HMO + 1% OO + 3% SO (%)	Group 5 16% HMO + 4% SO (%)	Group 6 10% HSOY + 9% OO + 1% SO (%)	Group 7 20% PALM (%)
Dietary ratio: 18:2/*trans*	0.06	0.15	0.25	0.34	0.44	0.25	—
PC							
16:0	16.4 ± 0.3	15.2 ± 0.4	15.0 ± 0.4	13.9 ± 0.6	14.0 ± 0.4	13.0 ± 0.6	19.3 ± 0.5
16:1 *t*	1.0 ± 0.0	1.1 ± 0.2	1.2 ± 0.1	1.2 ± 0.0	1.1 ± 0.0	0.6 ± 0.1	—
16:1 *c*	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.8 ± 0.0	0.8 ± 0.1	0.4 ± 0.0	0.5 ± 0.1
18 ald.	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
18:0	31.2 ± 0.9	27.6 ± 2.0	23.7 ± 1.4	21.6 ± 0.7	23.5 ± 0.6	20.4 ± 1.7	25.3 ± 0.6
18:1 *t*	5.4 ± 0.3	7.0 ± 0.3	7.7 ± 0.2	8.3 ± 0.6	8.3 ± 0.1	14.9 ± 1.0	—
18:1 *c*	11.0 ± 0.6	9.1 ± 0.5	7.8 ± 0.8	6.5 ± 0.3	4.8 ± 0.1	8.8 ± 0.1	7.8 ± 0.6
18:2 *c,t/t,c*	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.0	0.3 ± 0.2	0.3 ± 0.0	—
18:2 *c,c*	6.8 ± 0.4[c]	8.5 ± 0.4[c]	10.0 ± 1.4[c,d]	12.0 ± 1.0[c,d]	11.3 ± 0.5[c,d]	6.0 ± 0.6	4.8 ± 0.4
18:3/20:1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:3(n-9)	4.3 ± 0.3	1.5 ± 0.4	0.6 ± 0.2	0.5 ± 0.2	0.1 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
20:3(n-6)	2.4 ± 0.1[c,d]	2.1 ± 0.1[c,d]	1.6 ± 0.4[c,d]	1.7 ± 0.2[c,d]	1.1 ± 0.2[c]	0.8 ± 0.1	0.6 ± 0.2
20:4(n-6)	12.0 ± 1.0[c,d]	19.9 ± 0.7[c,d]	25.5 ± 0.8[c]	27.6 ± 1.8[c]	29.6 ± 0.3[c]	28.4 ± 0.7[c]	34.9 ± 0.6
22:4(n-6)	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0
22:5(n-6)	1.1 ± 0.2[c]	1.1 ± 0.2[c]	1.0 ± 0.3[c]	0.7 ± 0.1[c]	0.8 ± 0.1[c]	0.8 ± 0.2[c]	2.6 ± 0.5
22:5(n-3)	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
22:6(n-3)	2.9 ± 0.2	2.3 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.3 ± 0.0	3.4 ± 0.3	2.4 ± 0.1
PE							
16 ald.	0.3 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	0.2 ± 0.0	0.7 ± 0.1
16:0	13.7 ± 0.2	12.3 ± 0.3	11.2 ± 0.4	11.0 ± 0.4	10.6 ± 0.3	9.4 ± 0.4	17.2 ± 0.2
16:1 *t*	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	0.3 ± 0.0	—
16:1 *c*	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
18 ald.	0.5 ± 0.2	0.7 ± 0.1	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	0.3 ± 0.1
18:0	22.9 ± 1.5	21.2 ± 1.5	20.0 ± 0.3	18.3 ± 1.0	18.7 ± 0.5	14.4 ± 0.3	23.9 ± 0.7
18:1 *t*	7.5 ± 0.5	9.1 ± 0.3	9.8 ± 0.2	10.6 ± 0.3	10.2 ± 0.3	17.4 ± 0.4	—
18:1 *c*	7.4 ± 0.3	6.5 ± 0.3	6.2 ± 0.3	5.9 ± 0.3	4.7 ± 0.4	6.5 ± 0.3	5.7 ± 0.6
18:2 *c,t/t,c*	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	—
18:2 *c,c*	4.0 ± 0.3	5.4 ± 0.5[c]	7.1 ± 1.0[c,d]	9.8 ± 0.6[c,d]	9.0 ± 0.3[c,d]	3.7 ± 0.4	2.8 ± 0.2
18:3/20:1	1.3 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	1.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
20:3(n-9)	2.7 ± 0.3	0.6 ± 0.2	0.2 ± 0.0	0.1 ± 0.1	tr.	tr.	0.1 ± 0.0
20:3(n-6)	1.5 ± 0.1[c,d]	1.2 ± 0.1[c,d]	0.9 ± 0.2[c,d]	1.0 ± 0.2[c,d]	0.8 ± 0.1[c,d]	0.5 ± 0.0	0.3 ± 0.1
20:4(n-6)	22.2 ± 1.3[c,d]	29.4 ± 0.5[c,d]	32.2 ± 0.7	32.1 ± 0.8	33.6 ± 0.3	32.7 ± 0.6	33.6 ± 0.6
22:4(n-6)	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	1.2 ± 0.1
22:5(n-6)	1.7 ± 0.3[c]	2.1 ± 0.4[c]	1.9 ± 0.4[c]	1.4 ± 0.3[c]	2.1 ± 0.4[c]	1.6 ± 0.5[c]	5.6 ± 0.6
22:5(n-3)	0.5 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.6 ± 0.0	0.5 ± 0.0
22:6(n-3)	8.2 ± 0.5	6.3 ± 0.4	4.8 ± 0.3	3.8 ± 0.3	3.7 ± 0.1	9.4 ± 0.5	6.7 ± 0.4
Phospholipids mg/g tissue	40.2 ± 2.4	41.2 ± 5.0	40.0 ± 4.7	42.7 ± 0.9	42.3 ± 1.2	40.1 ± 1.0	40.7 ± 1.5

[a]Averages of 4 different pools ± Standard error of the mean.

[b]Abbreviations as in Materials and Methods.

[c]Significantly different than group 7. $P < 0.05$.

[d]Significantly different than group 6. $P < 0.05$.

TABLE 3

Triglycerides from Rat Liver, Heart and Adipose Tissue[a]

Fatty acid	Dietary fat						
	Group 1 16% HMO + 4% OO (%)	Group 2 16% HMO + 3% OO + 1% SO (%)	Group 3 16% HMO + 2% OO + 2% SO (%)	Group 4 16% HMO + 1% OO + 3% SO (%)	Group 5 16% HMO + 4% SO (%)	Group 6 10% HSOY + 9% OO + 1% SO (%)	Group 7 20% PALM (%)
Dietary ratio: 18:2/*trans*	0.06	0.15	0.25	0.34	0.44	0.25	—
Liver							
16:0	29.4 ± 1.4	26.7 ± 0.8	27.0 ± 3.8	23.6 ± 0.7	22.9 ± 0.7	21.4 ± 1.1	31.9 ± 0.4
16:1 *t*	3.4 ± 0.3	3.7 ± 0.1	3.8 ± 0.4	4.2 ± 0.2	4.1 ± 0.2	2.9 ± 0.2	—
16:1 *c*	4.7 ± 0.2	4.3 ± 0.4	4.0 ± 0.6	4.0 ± 0.4	3.9 ± 0.3	1.9 ± 0.4	3.0 ± 0.5
18:0	2.5 ± 0.1	1.9 ± 0.2	2.1 ± 0.3	1.8 ± 0.1	1.8 ± 0.2	1.4 ± 0.2	1.7 ± 0.2
18:1 *t*	3.9 ± 0.4	4.5 ± 0.2	4.5 ± 0.5	5.4 ± 0.4	5.2 ± 0.4	7.1 ± 0.4	—
18:1 *c*	47.0 ± 0.9	43.6 ± 0.8	36.2 ± 2.1	32.0 ± 0.6	24.7 ± 0.7	51.5 ± 0.5	49.9 ± 0.6
18:2 *c,t/t,c*	1.3 ± 0.4	0.9 ± 0.3	0.7 ± 0.4	1.1 ± 0.3	1.3 ± 0.1	1.7 ± 0.2	—
18:2 *c,c*	2.5 ± 0.4[b,c]	8.7 ± 0.4	14.3 ± 1.1[b,c]	20.3 ± 0.6[b,c]	26.3 ± 1.2[b,c]	9.7 ± 0.9	10.6 ± 0.4
18:3(n-3)	0.7 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.2 ± 0.0	1.3 ± 0.0	1.2 ± 0.1	0.3 ± 0.0
20:1	2.4 ± 0.2	2.6 ± 0.1	3.3 ± 0.3	3.4 ± 0.2	3.3 ± 0.3	—	—
20:4 + 22:1	0.4 ± 0.1	0.7 ± 0.1	1.1 ± 0.3	1.1 ± 0.1	1.6 ± 0.2	0.6 ± 0.2	1.1 ± 0.4
TG mg/g tissue	6.3 ± 1.9	6.1 ± 0.5	7.6 ± 3.3	8.8 ± 1.3	8.4 ± 1.2	10.4 ± 2.7	11.0 ± 2.6
Heart							
14:0	4.6 ± 0.2	4.6 ± 0.1	4.7 ± 0.1	4.8 ± 0.0	4.6 ± 0.1	1.1 ± 0.2	1.5 ± 0.1
16:0	23.0 ± 0.8	23.2 ± 0.2	22.2 ± 0.6	20.6 ± 0.2	20.3 ± 0.7	17.2 ± 0.6	35.5 ± 1.0
16:1 *t*	3.6 ± 0.1	3.7 ± 0.1	3.5 ± 0.0	3.7 ± 0.1	3.7 ± 0.1	1.0 ± 0.1	—
16:1 *c*	5.0 ± 0.1	4.8 ± 0.1	4.6 ± 0.2	4.6 ± 0.1	4.4 ± 0.3	1.4 ± 0.2	2.5 ± 0.3
18:0	4.3 ± 0.4	3.3 ± 1.1	4.5 ± 0.1	4.4 ± 0.1	4.9 ± 0.4	3.6 ± 0.1	5.1 ± 0.2
18:1 *t*	6.3 ± 0.2	5.0 ± 1.7	6.7 ± 0.0	7.2 ± 0.1	7.0 ± 0.4	13.6 ± 0.7	—
18:1 *c*	41.0 ± 1.2	37.0 ± 0.2	31.2 ± 0.5	27.6 ± 0.6	22.5 ± 0.8	49.3 ± 2.0	43.2 ± 0.7
18:2 *c,t/t,c*	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	1.5 ± 0.1	—
18:2 *c,c*	3.2 ± 0.1[b,c]	6.4 ± 0.1[b]	9.9 ± 0.2[b,c]	13.6 ± 0.3[b,c]	17.0 ± 0.6[b,c]	6.7 ± 0.5[b]	8.6 ± 0.2
20:1 *t*	1.2 ± 0.3	0.9 ± 0.2	1.7 ± 0.1	1.9 ± 0.2	1.4 ± 0.3	—	—
20:1 *c*	6.4 ± 0.5	6.1 ± 0.2	6.2 ± 0.1	6.6 ± 0.1	6.6 ± 0.3	—	—
20:4 ± 22:1	1.2 ± 0.1	1.2 ± 0.0	2.2 ± 0.2	2.1 ± 0.1	2.7 ± 0.5	0.3 ± 0.0	0.4 ± 0.1
TG mg/g tissue	1.7 ± 0.1	1.7 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	1.8 ± 0.1	2.4 ± 0.3	2.2 ± 0.1
Adipose tissue							
14:0	4.5 ± 0.1	4.8 ± 0.1	5.0 ± 0.1	5.2 ± 0.0	5.1 ± 0.1	0.4 ± 0.0	0.9 ± 0.0
14:1 *t*	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	tr.	—
14:1 *c*	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	tr.	0.1 ± 0.0
16:0	21.0 ± 0.2	20.2 ± 0.2	19.5 ± 0.4	18.8 ± 0.3	18.7 ± 0.5	13.0 ± 0.2	32.7 ± 0.3
16:1 *t*	4.4 ± 0.1	4.8 ± 0.2	4.6 ± 0.1	4.8 ± 0.0	4.9 ± 0.1	1.2 ± 0.1	—
16:1 *c*	7.1 ± 0.1	6.9 ± 0.1	6.6 ± 0.2	6.5 ± 0.2	5.6 ± 0.3	2.1 ± 0.2	3.8 ± 0.2
18:0	3.0 ± 0.1	3.2 ± 0.1	3.4 ± 0.2	3.5 ± 0.2	3.3 ± 0.1	2.3 ± 0.1	3.2 ± 0.2
18:1 *t*	6.8 ± 0.2	7.1 ± 0.2	6.8 ± 0.1	7.2 ± 0.2	7.4 ± 0.3	15.6 ± 1.2	—
18:1 *c*	43.0 ± 0.6	38.8 ± 0.3	32.9 ± 0.5	28.3 ± 0.5	23.0 ± 0.4	55.8 ± 1.3	49.6 ± 0.3
18:2 *c,t/t,c*	0.9 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.7 ± 0.1	—
18:2 *c,c*	2.4 ± 0.1[b,c]	6.1 ± 0.1[b,c]	10.9 ± 0.2[b,c]	15.0 ± 0.1[b,c]	20.0 ± 0.3[b,c]	7.3 ± 0.3[b]	9.5 ± 0.2
20:1 *t*	0.9 ± 0.1	1.0 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	1.2 ± 0.3	—	—
20:1 *c*	3.8 ± 0.4	3.5 ± 0.5	4.6 ± 0.3	4.7 ± 0.4	4.6 ± 0.4	—	0.1 ± 0.0
20:4 + 22:1	0.3 ± 0.1	0.3 ± 0.2	0.6 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	—	—

[a]Averages of 4 different pools ± standard error of the mean.

[b]Significantly different than group 7. $P < 0.05$.

[c]Significantly different than group 6. $P < 0.05$.

to changes in the fatty acid composition of the microsomal membrane possibly affecting the membrane-bound desaturases or their associated electron transport chain. The importance of the membrane effect is substantiated by the observation that microsomes from rats fed HMO have lower desaturase activities than microsomes from rats fed partially hydrogenated vegetable oils, although the microsomes contain no 20:1 or 22:1 isomers (16).

TABLE 4

Fatty Acids in Phosphatidylcholines and Phosphatidylethanolamines and Total Phospholipid Content in Rat Heart[a]

	Dietary fat						
Fatty acid	Group 1 16% HMO + 4% OO (%)	Group 2 16% HMO + 3% OO + 1% SO (%)	Group 3 16% HMO + 2% OO + 2% SO (%)	Group 4 16% HMO + 1% OO + 3% SO (%)	Group 5 16% HMO + 4% SO (%)	Group 6 10% HSOY + 9% OO + 1% SO (%)	Group 7 20% PALM (%)
Dietary ratio: 18:2/*trans*	0.06	0.15	0.25	0.34	0.44	0.25	—
PC							
16:0	11.1 ± 0.5	11.0 ± 0.4	10.5 ± 0.4	10.1 ± 0.1	10.6 ± 0.4	12.2 ± 0.4	14.3 ± 0.3
16:1 *t*	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	—
16:1 *c*	0.7 ± 0.0	0.8 ± 0.3	0.6 ± 0.1	1.1 ± 0.2	0.7 ± 0.2	0.6 ± 0.1	0.2 ± 0.1
18:0	28.5 ± 0.2	25.4 ± 1.3	22.7 ± 0.4	24.7 ± 0.5	26.3 ± 1.2	18.4 ± 0.3	30.2 ± 0.4
18:1 *t*	5.2 ± 0.4	5.9 ± 0.3	5.6 ± 0.1	6.3 ± 0.3	6.1 ± 0.2	12.4 ± 0.3	—
18:1 *c*	12.6 ± 0.3	10.6 ± 0.3	9.1 ± 0.2	8.2 ± 0.4	7.0 ± 0.3	12.5 ± 0.3	8.1 ± 0.2
18:2 *c,t/t,c*	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.0	0.3 ± 0.0	—
18:2 *c,c*	9.1 ± 0.5[b,c]	10.3 ± 1.0[b,c]	9.9 ± 0.7[b,c]	11.5 ± 0.8[b,c]	10.7 ± 0.5[b,c]	5.6 ± 0.2	4.5 ± 0.1
18:3(n-3)	—	—	—	—	—	0.2 ± 0.0	0.1 ± 0.0
20:1 *t*	0.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	—	—
20:1 *c*	1.3 ± 0.2	1.0 ± 0.2	1.2 ± 0.3	1.1 ± 0.1	1.0 ± 0.0	—	—
20:3(n-9)	2.2 ± 0.1	0.3 ± 0.2	tr.	—	—	0.1 ± 0.1	0.2 ± 0.0
20:3(n-6)	1.6 ± 0.1[b,c]	0.8 ± 0.2[b,c]	0.7 ± 0.2[b,c]	0.5 ± 0.1[b]	0.4 ± 0.1[b]	0.2 ± 0.0	0.1 ± 0.0
20:4(n-6)	20.4 ± 0.9[b,c]	28.6 ± 0.7[b,c]	32.9 ± 0.8[b]	31.5 ± 1.2[b]	31.3 ± 0.7[b]	31.2 ± 0.9[b]	35.8 ± 0.2
22:4(n-6)	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.6 ± 0.0	0.9 ± 0.0
22:5(n-6)	0.5 ± 0.1[b]	0.5 ± 0.2[b]	0.8 ± 0.1[b]	0.7 ± 0.1[b]	1.0 ± 0.2[b]	0.9 ± 0.1[b]	2.6 ± 0.2
22:5(n-3)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
22:6(n-3)	1.8 ± 0.2	1.4 ± 0.2	1.2 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	2.4 ± 0.1	1.5 ± 0.0
PE							
16 ald.	5.7 ± 0.6	1.0 ± 1.0	1.7 ± 0.4	0.2 ± 0.1	2.8 ± 0.5	1.7 ± 0.6	3.7 ± 1.5
16:0	4.2 ± 0.1	7.6 ± 0.0	6.0 ± 0.2	7.7 ± 0.4	5.8 ± 0.7	4.4 ± 0.2	7.5 ± 0.5
16:1 *t*	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	—
16:1 *c*	0.4 ± 0.1	0.6 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	2.4 ± 0.2	2.8 ± 0.8
18:0 ald.	3.7 ± 0.4	0.9 ± 0.4	1.7 ± 0.2	0.8 ± 0.2	2.4 ± 0.2	1.0 ± 0.4	1.9 ± 0.6
18:1 ald.	5.4 ± 0.8	1.0 ± 0.6	1.8 ± 0.3	0.7 ± 0.3	2.6 ± 0.2	2.7 ± 1.4	—
18:0	20.9 ± 2.1	23.2 ± 1.5	26.2 ± 1.8	22.0 ± 0.4	21.2 ± 1.1	18.3 ± 0.6	25.1 ± 0.6
18:1 *t*	4.9 ± 0.1	7.3 ± 0.1	5.8 ± 0.3	7.3 ± 0.3	6.6 ± 0.4	15.2 ± 0.8	—
18:1 *c*	7.9 ± 0.0	9.8 ± 0.2	7.5 ± 0.7	9.3 ± 0.4	6.4 ± 0.3	10.2 ± 0.5	5.9 ± 0.5
18:2 *c,t/t,c*	0.2 ± 0.0	0.7 ± 0.0	0.4 ± 0.0	0.9 ± 0.1	0.4 ± 0.1	1.2 ± 0.2	—
18:2 *c,c*	3.7 ± 0.4	3.4 ± 0.1[c]	4.0 ± 0.3[b,c]	4.8 ± 0.4[b,c]	5.3 ± 0.2[b,c]	1.5 ± 0.3	2.0 ± 0.1
18:3(n-3)	—	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	—
20:1 *t*	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	—	—
20:1 *c*	1.5 ± 0.1	1.7 ± 0.1	1.7 ± 0.2	1.9 ± 0.2	1.7 ± 0.1	—	—
20:3(n-9)	0.7 ± 0.1	0.1 ± 0.0	—	—	—	—	—
20:3(n-6)	0.6 ± 0.1[b,c]	0.3 ± 0.0[b,c]	0.3 ± 0.0[b,c]	0.3 ± 0.1[c]	0.2 ± 0.0[c]	0.1 ± 0.0	0.1 ± 0.0
20:4(n-6)	25.4 ± 0.4[b]	27.3 ± 0.3[b,c]	27.7 ± 1.4[b,c]	29.2 ± 0.7[b,c]	27.8 ± 0.3[b,c]	23.1 ± 0.5	21.3 ± 0.6
22:4(n-6)	0.4 ± 0.0	0.8 ± 0.0	1.2 ± 0.1	1.4 ± 0.1	1.9 ± 0.3	1.7 ± 0.0	2.6 ± 0.1
22:5(n-6)	2.3 ± 0.2[b]	3.3 ± 0.3[b]	4.2 ± 0.5[b]	4.3 ± 0.2[b]	6.6 ± 0.8[b]	4.2 ± 0.1[b]	15.8 ± 1.1
22:5(n-3)	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.7 ± 0.0	0.9 ± 0.1
22:6(n-3)	9.4 ± 0.0	8.4 ± 0.9	6.5 ± 0.1	5.7 ± 0.2	5.3 ± 0.2	10.9 ± 0.2	8.7 ± 0.2
Phospholipid mg/g tissue	26.1 ± 0.9	25.9 ± 0.9	26.4 ± 1.0	25.5 ± 0.8	25.6 ± 1.2	24.6 ± 1.4	27.8 ± 0.9

[a]Averages of 4 different pools ± standard error of the mean.

[b]Significantly different than group 7. $P < 0.05$.

[c]Significantly different than group 6. $P < 0.05$.

TABLE 5

Catalase Activity ($min^{-1} \times mg\ protein^{-1}$) in Rat Liver Fractions Enriched with Peroxisomes

	Dietary fat						
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
	16% HMO + 4% OO (%)	16% HMO + 3% OO + 1% SO (%)	16% HMO + 2% OO + 2% SO (%)	16% HMO + 1% OO + 3% SO (%)	16% HMO + 4% SO (%)	10% HSOY + 9% OO + 1% SO (%)	20% PALM (%)
Dietary 18:2*trans*	0.06	0.15	0.25	0.34	0.44	0.25	—
	91.7 ± 5.3[a,b]	97.7 ± 2.6[a,b]	91.1 ± 2.4[a,b]	84.5 ± 2.6[a,b]	80.7 ± 2.3[a]	73.1 ± 2.7[a]	54.5 ± 2.6

[a]Significantly different than group 7. $P < 0.05$.

[b]Significantly different than group 6. $P < 0.05$.

Previous feeding studies with rats have also established that rats fed partially hydrogenated vegetable or marine fats supplemented with vegetable oils have lower levels of long chain PUFA than rats fed vegetable oils providing either a considerably higher level of dietary linoleic acid (14,15) or similar level of dietary linoleic acid (25,26).

In the present experiment, we investigated if, by increasing the dietary level of 18:2(n-6) relative to the contents of isomeric fatty acids and far above the level of a reference group, it would be possible to increase the levels of 20:4(n-6) in the liver and heart phospholipids to those of the reference group not fed isomeric fatty acids. Furthermore, a group was fed HSOY to compare effects of chain lengths of isomeric fatty acids.

This experiment demonstrates that rats fed partially hydrogenated fats have lower content of 20:4(n-6) in liver PC than a reference group fed unhydrogenated fats with a similar dietary level of 18:2(n-6). Furthermore, it does not seem possible to augment the formation of 20:4(n-6) in the HMO-fed rats to the level of the reference group by increasing the dietary levels of 18:2 within the limits of this experiment. Compared with the HMO groups, the HSOY-fed rats have a higher formation of 20:4, which is consistent with our previous results (14). The lower formation of 20:4(n-6) in HMO-fed animals is paralleled by increased accumulations of 18:2(n-6) in the phospholipids, indicating that the total liver pool of 18:2 is enlarged and pointing to an effect of dietary fats on the desaturation of linoleic acid. This view is supported by the substantial channeling of 18:2 into TG in liver, heart and adipose tissue. However, we find lower incorporation of isomeric fatty acids in the phospholipids of the HMO-fed rats than of the HSOY-fed rats. Furthermore, only isomeric- 16:1 and 18:1 fatty acids are deposited, although the HMO contains large amounts of 20:1 and 22:1 isomers. The absence of these fatty acid isomers from the liver may be explained by the peroxisomal β-oxidation induced by long chain fatty acids. This is evidenced by the increased catalase activity in the HMO groups compared with the HSOY and the PALM group. The significance of peroxisomal β-oxidation in the degradation of 20:4(n-6) appears not to have been elucidated in vivo, but in vitro experiments seem to confirm this pathway (27).

The PUFA contents of PE in liver are less affected by dietary fats than PC, which is in agreement with a recent report (28). Since a higher unsaturation is generally found in PE than in PC, this mainly indicates that the mechanisms for maintaining the unsaturation of PE by selective reacylation of PE formed de novo or by channeling less unsaturated molecular species into PC is also operative in rats fed partially hydrogenated oils. At low dietary levels of 18:2(n-6), the conversion of 18:3(n-3) into 22:5(n-3) and 22:6(n-3) is more efficient in agreement with the findings of Charnock et al. (29).

In the heart, the fatty acid profiles are generally more unsaturated than in the liver. The PUFA may be formed from 18:2 taken from chylomicrons or from 18:2 and 20:4 taken from very low density lipoproteins (VLDL) secreted from the liver. The fatty acid composition of VLDL-TG is similar to liver TG (30). The considerable accumulation of 18:2 observed in liver TG may, in part, explain that even at low dietary linoleic acid levels the HMO-fed rats have high (n-6) PUFA levels in the heart phospholipids. Enhanced activity of lipoprotein lipase following intake of docosenoic acids (31) may accentuate the uptake of fatty acids of hepatic or dietary origin in the extrahepatic tissues. Just as in the liver, the heart phospholipids have low levels of *trans* fatty acids in the HMO-fed rats and only trace amounts of *trans*-20:1 and *trans*-22:1 are found in agreement with the findings of Conacher et al. (6). This reflects the efficiency of peroxisomal β-oxidation of 20:1 and 22:1 fatty acids, as described by Christiansen et al. (32). It thus appears unlikely that the isomeric fatty acids in the organ phospholipids per se constitute the factor that affects the conversion of (n-6) PUFA. In heart PC, the effects of HMO on the formation of 20:4 are significantly smaller than observed for the liver. This agrees with the observation that the heart levels of 18:2 show less dependence on the dietary levels than do the liver levels. Compared with the PALM group, however, a substantial decrease in 20:4 is paralleled by an increase in 18:2, indicating a decreased conversion of 18:2 into long chain PUFA.

Heart PE, as well as PC, is little affected by the dietary levels of linoleic acid in the HMO-fed rats. In the PALM group, however, a substantially higher level of 22:5(n-6) is found in PE, compared with HSOY-fed rats, as found by Royce and Holmes (33). In the HMO groups, the 22:5(n-6) is low and the 20:4(n-6) is high compared with the PALM group, in agreement with a previous report

in which partially hydrogenated herring oil, rich in *cis*-20:1 and *cis*-22:1 relative to *trans*, decreased the formation of 22:5(n-6), but partially hydrogenated capelin oil with a high *trans/cis* ratio did not (15). It is thus possible that the formation of 22:5(n-6) by Δ4-desaturase may be affected by the presence of certain long chain isomers in the heart. Retroconversion of 22:4(n-6) and 22:6(n-3) has been shown to be increased in isolated liver cells from rats reared on HMO diets (34). It is thus possible that this also influences the 22:5 levels.

The linear relationship between the dietary 18:2 levels and that of the heart TG contrasts with the constant levels of (n-6) PUFA in PC and PE of the HMO-fed rats, indicating that the PUFA metabolism, in general, is under extreme control in the myocardium over a wide range of dietary supply of 18:2, as pointed out by Gibson et al. (35), making it especially interesting to note that HMO can perturb the PUFA profiles leading to lower levels of 20:4(n-6) and 22:5(n-6) in HMO- and HSOY-fed rats than in PALM-fed rats. At low dietary and cardiac levels of 18:2, this acid is selectively channeled into a pool available for formation of 20:4(n-6) and incorporated into phospholipids. At high dietary levels of linoleic acid, apparently the 18:2 is stored in TG unavailable for PUFA production, although the levels of long chain PUFA are lower than in the PALM group.

Our comprehension of the physiological implications of a simultaneous incorporation of isomeric fatty acids into biological membranes combined with a reduced formation of long chain PUFA is, so far, quite poor due to the lack of reliable experimental models to evaluate changes in biological membranes by physical or biochemical parameters. It has been demonstrated in vitro (36) that increased fluidity of the microsomal membranes is associated with decreased activity of desaturases, which supports the concept of the microsomal membrane as a self-regulating system based on interaction between "fluidity" and desaturase activity (37), which could be operative under a wide range of dietary conditions (35). On the other hand, numerous investigators have demonstrated that intake of partially hydrogenated fats, even when supplemented with adequate levels of linoleic acid, may reduce the contents of PUFA and, thus, the fluidity, indicating that the regulation of fatty acid composition may be perturbed by external factors such as certain dietary fatty acids. Whether this effect is exerted on the environment of the desaturase or eventually on the synthesis of desaturase remains to be clarified (38). In the evaluation of the possible effects of a prolonged intake of partially hydrogenated fats, it should, however, be pointed out that multigeneration feeding studies with rats so far have not proven any harmful effects of partially hydrogenated vegetable fats, as long as linoleic acid is supplied (39). This could indicate that even major changes in the composition of biological membranes may not significantly affect their biological efficiency. However, similar experiments have not been performed with partially hydrogenated marine oils.

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REFERENCES

1. Carpenter, D.L., and Slover, H.T. (1973) *J. Am. Oil Chem. Soc. 50,* 372-376.
2. Druckrey, F., Høy, C.-E., and Hølmer, G. (1985) *Fette Seifen Anstrichm. 87,* 350-55.
3. Wood, R. (1979) *Lipids 14,* 975-982.
4. Reichwald-Hacker, I., Ilseman, K., and Mukherjee, K.D. (1979) *J. Nutr. 109,* 1051-1056.
5. Høy, C.-E., and Hølmer, G. (1979) *Lipids 14,* 727-733.
6. Conacher, H.B.S., Page, B.D., and Beare-Rogers, J.L. (1973) *Lipids 8,* 256-258.
7. Høy, C.-E. (1977) Ph.D. Thesis, Technical University of Denmark, Lyngby, Denmark.
8. Ohlrogge, J.B., Emken, E.A., and Gulley, R.M. (1981) *J. Lipid Res. 22,* 955-960.
9. Heckers, H., Korner, M., Tuschen, T.W.L., and Melcher, F.W. (1977) *Atherosclerosis 28,* 389-398.
10. Mahfouz, M.M., Johnson, S., and Holman, R.T. (1980) *Lipids 15,* 100-107.
11. Mahfouz, M.M., Johnson, S., and Holman, R.T. (1981) *Biochim. Biophys. Acta 663,* 58-68.
12. Anderson, R.L., Fullmer, C.S., Jr., and Hollenbach, E.J. (1975) *J. Nutr. 105,* 393-400.
13. Privett, O.S., Phillips, F., Shimazaki, H., Nozawa, T., and Nickell, E.C. (1977) *Am. J. Clin. Nutr. 30,* 1009-1017.
14. Hølmer, G., Høy, C.-E., and Kirstein, D. (1982) *Lipids 17,* 585-593.
15. Blomstrand, R., and Svensson, L. (1983) *Lipids 18,* 151-170.
16. Kirstein, D., Høy, C.-E., and Hølmer, G. (1983) *Br. J. Nutr. 50,* 749-756.
17. Svensson, L. (1983) *Lipids 18,* 171-178.
18. Aaes-Jørgensen, E., and Hølmer, G. (1969) *Lipids 4,* 501-506.
19. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
20. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) *Lipids 5,* 494-496.
21. Høy, C.-E., and Hølmer, G. (1981) *Lipids 16,* 102-108.
22. Hicks, C.R. (1973) *Fundamental Concepts in the Design of Experiments,* 3rd ed., pp. 35-36, Holt, Rinehart, and Winston, New York.
23. Marcel, Y.L., Christiansen, K., and Holman, R.T. (1968) *Biochim. Biophys. Acta 164,* 25-34.
24. Hassam, A.G., Sinclair, A.J., and Crawford, M.A. (1975) *Lipids 10,* 417-420.
25. Mahfouz, M.M., Smith, T.L., and Kummerow, F.A. (1984) *Lipids 19,* 214-222.
26. Blomstrand, R., Diczfalusy, U., Sisfontes, L., and Svensson, L. (1985) *Lipids 20,* 283-295.
27. Hiltunen, J.K., Karki, T., Hassinen, I.E., and Osmundsen, H. (1986) *J. Biol. Chem. 261,* 16484-16493.
28. Lawson, L.D., Hill, E.G., and Holman, R.T. (1985) *Lipids 20,* 262-267.
29. Charnock, J.S., Abeywardena, M.Y., McMurchie, E.J., and Russell, G.R. (1984) *Lipids 19,* 206-213.
30. Thomassen, M.S., Strøm, E., Christiansen, E.N., and Norum, K.R. (1979) *Lipids 14,* 58-65.
31. Hülsmann, W.C., Geelhood-Mieras, M.M., Jansen, H., and Houtsmüller, U.M.T. (1979) *Biochim. Biophys. Acta 572,* 183-187.
32. Christiansen, E.N., Thomassen, M.S., Christiansen, R.Z., Osmundsen, H., and Norum, K.R. (1979) *Lipids 14,* 829-835.
33. Royce, S.M., and Holmes, R.P. (1983) *Biochim. Biophys. Acta 792,* 371-375.
34. Hagve, T.-A., and Christophersen, B. (1986) *Biochim. Biophys. Acta 6,* 165-173.
35. Gibson, R.A., McMurchie, E.J., Charnock, J.S., and Kneebone, G.M. (1984) *Lipids 19,* 942-951.
36. Garda, H.A., and Brenner, R.R. (1984) *Biochim. Biophys. Acta 769,* 160-170.
37. Brenner, R.R. (1984) *Prog. Lipid Res. 23,* 69-96.
38. Pugh, E.L., and Kates, M. (1984) *Lipids 19,* 60-63.
39. Alfin-Slater, R.B., Wells, P., Aftergood, L., and Melnick, D. (1973) *J. Am. Oil Chem. Soc. 50,* 479-484.

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Serum Fatty Acid Proportions Are Altered During the Year Following Acute Epstein-Barr Virus Infection

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Because abnormal serum fatty acid (FA) proportions had been found at three months after infectious mononucleosis (IM) in a pilot study, serum total FA profiles of 20 normal college students were measured at monthly intervals for one year following an acute Epstein-Barr virus (EBV) infection. Below normal proportions of arachidonic acid and a reversal of the usual serum ratio of linoleic and oleic acids were maximal during the third month after acute IM. These FA abnormalities coincided with the symptom of increased physical malaise, despite apparent clinical recovery, common after IM. Persistence of low linoleic acid content beyond six months postinfection occurred in all seven students who showed continued clinical symptoms. Estimation of FA enzyme activities over the post-IM year suggested that FA elongation function was normal, but that FA desaturation enzyme activities were lower than normal, particularly early after EBV infection. An inability of the host to normalize the serum total linoleic/oleic acid ratio may parallel a delayed recovery from EBV infection and may offer insight into its pathogenesis.

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Common intermittent fatigue after acute primary Epstein-Barr virus (EBV) clinical infection, termed infectious mononucleosis (IM), suggests that host systems are severely stressed by the infection (1). A temporal relationship between altered serum fatty acid (FA) patterns, described after acute and chronic illnesses (2-6), confirms a relationship between altered lipid metabolism and disease. Maintenance of normal serum-FA content is essential to homeostasis and health. The ratio or percentage of individual FA in normal fasting serum remains fairly constant within age boundaries (7). Serum FA are substrates for many cellular systems in the body. For example, serum polyunsaturated arachidonic acid is a precursor for important immunoregulatory substances, prostaglandins and leukotrienes that increase dramatically in disease (8). Long-chain polyunsaturated FA have additional roles in maintaining fluidity and stability of membranes, particularly in the vascular system (9). FA serve also as substrates for red-muscle mitochondrial oxidative phosphorylation, and neuronal (10) metabolism. Serum-FA measurements may reflect an imbalance or inadequacy of FA amounts available for lipoprotein synthesis. Measurements over time may indicate possible metabolic FA-tissue turnover events. Recent characterization of desaturation and elongation metabolic pathways of FA have led to understanding the multiple roles of FA in health and disease (11-15).

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Abbreviations: FA, fatty acid; Ig, immunoglobulin; ANOVA, analysis of variance; EBV, Epstein-Barr virus; GLC, gas liquid chromatography; IM, infectious mononucleosis; L/O ratio, linoleic to oleic acid ratio.

Acute EBV infection, as any acute illness, alters host lipid metabolism by direct utilization of host lipids and by destruction of host cells (6,16). Host lipid-related metabolic systems can be directly affected in production of endotoxins, in activation of lipase or other host macrophage lysosomal enzymes, or in release of host mediators such as pyrogens, interferons and other immunoregulatory substances (10,13,16). Secondary or indirect effects of acute illness on host lipids may include decreased dietary intake of fats from anorexia, possible decreased intestinal absorption of fats, and increased rates of host lipolysis for the metabolic demands of fever and replacement of tissues. The acute toxic state with prostration also leads to altered rates of liver lipid synthesis and lowered fat utilization by peripheral tissues (6,16). Although important in understanding the immediate response to EBV and other infections, the majority of these stresses on host lipid metabolism appear to resolve after the first month of infectious mononucleosis illness, as fever disappears and clinical recovery occurs.

EBV infection appears rather unique among acute viral infections in that it leads frequently to a delayed return to robust health (1,8,12,13). Because we wished to focus on the prolonged period of weakness and malaise after IM, we began our study near the end of the first month after acute IM. The "onset" of EBV infection was considered to be the date of a positive monospot test with a characteristic acute IM-type of illness in healthy college students. Although it is possible that a subset of more severely-affected post-IM students was selected by our method, sample bias was minimized by a payment and by the encouragement of cooperation in the study by health clinic personnel. At our first fasting blood sampling, the student was afebrile, ambulatory, again able to eat a normal diet and had recovered sufficiently to return to classes. We report here total FA profiles of 20 normal students followed monthly thereafter for one year.

METHODS

Patient selection and sample preparation. Normal college students, 10 male and 10 female, aged 18-23, with an acute illness characterized by severe pharyngitis, generalized lymphadenopathy, fever, prostration and a positive heterophile antibody test, voluntarily participated. An elevated white blood cell count with lymphocytosis containing greater than 10% atypical mononuclear cells confirmed the diagnosis of Epstein-Barr virus (EBV) mononucleosis in all (1). Because we were interested in long-term results of EBV after the acute illness, the first blood specimen was drawn after confirmation of the diagnosis and agreement for enrollment in the program. Fasting blood samples were drawn at a regularly scheduled clinic visit occurring 2-4 wk after the positive heterophile test. Each subsequent visit was planned for 1 mo after the previous visit. No treatments were being given at the time of sampling. A dietary and systems review was taken at

each visit to monitor eating patterns and the possibility of physical complications. Age and sex-matched normals (20) who had no evidence of EBV infection for at least 4 yr were similarly followed. All laboratory personnel were blinded to the clinical diagnosis. Guidelines of the Human Subjects Research Committee of The Ohio State University were followed. No drugs, including aspirin, cortisone or similar products, were being taken 48 hr prior to blood drawing. Serum, separated immediately, was frozen at −20°C until extraction.

Serum total esterified fatty acid extraction for gas liquid chromatography (GLC). For extraction of total serum fatty acids, we adapted the method of Love et al. (2). Overnight stirring of 0.5 ml serum in 19 ml chloroform:methanol (2:1) containing BHT (1 μg/20 ml) was followed by adding 30 μg of 15:0 standard and 4 ml 0.1 M KCl. The lower phase was washed with 8 ml of upper phase solvents (containing chloroform:methanol:0.1 M KCl, 3:48:47, v/v/v). After drying the lower phase under N_2, the extract was hydrolyzed with 2 ml of 1 M KOH in 90% ethanol for 30 min, acidified with 5 ml H_2O containing 1.5 ml of 2 M HCl, extracted with 10 ml diethyl ether and taken to dryness under N_2. Methylation was accomplished by heating to 60°C in a dry bath for 10 min with 0.5 ml BF_3-methanol (Alltech Assoc., Deerfield, IL). After transfer to a separatory vial with hexane, and washing twice with 12 ml saturated NaCl, the methylated FA were dried with Na_2SO_4 crystals, dried under N_2 and kept until use at −20°C (2).

Detection of methylated lipid fractions and standards was performed on a Hewlett-Packard 5840-A Gas Liquid Chromatograph equipped with a dual hydrogen flame-ionization detector and an automatic integrator. A 30-m SP 2330 fused silica capillary column with a 0.20-μm-film thickness and 0.25 mm i.d. (Supelco, Bellfonte, PA) was used. The column was temperature programmed from 180°C to 240°C at 2°/min. The injection port temperature was 250°C, the detection temperature was 250°C, and the linear flow velocity was 10 ml/min, with the carrier gas being He and the split ratio being 1:100. Peaks on the chromatogram were identified by comparing retention times with reference standards (Supelco and Nuchek Prep, Elysian, MN). The percentage of each FA in each sample was calculated by the ratio of each FA area to the total area of the sample. The actual amount (mg/ml) of total FA in the sample was calculated from the amount of 15:0 standard (added at the onset of extraction) remaining in the final chromatogram and the dilution of the sample. Normal controls were included in daily runs. Variation in FA proportions among duplicate samples was less than 2%.

Detection of EBV-specific antibodies. Indirect immunofluorescence was used to examine the sera of the 7 post-EBV patients with linoleic:oleic acid (L/O) ratios of less than 1.20 for specific antibody content. To detect Immunoglobulin A with EBV specificity (EBV-IgA) and early antigens of EBV in the Immunoglobulin G (EBV-EA IgG) antibody fraction, the standard indirect immunofluorescent test was employed (16).

Statistical analyses. Average proportions of 20 patient serum FA, known as the FA profile, were compared with serial samples of fasting age-matched normals, drawn over the year. Analysis of variance (ANOVA) was used to calculate significant differences between the FA contents and estimated FA enzyme activities of the two groups at monthly intervals. The Bonferonni correction for multiple comparisons was made. A P value of <0.01 was considered significant (14). Metabolic indices, calculated according to the formulae of Holman and Johnson (15), estimated the activities of fatty acid elongation and desaturation enzymes at each month post infection. The average amount of FA products of each enzyme pathway in post-EBV serum was compared to the normal average amount to estimate enzyme activity in specific pathways.

RESULTS

Systems and dietary review. All patients expressed marked degrees of fatigue, exhaustion and mental discouragement during the first 3–5 mo after primary EBV infection. Unusual need for extra sleep was a common complaint which led to dropping classes by several students, and appeared more prominent in 4 of the 20 who had experienced slight jaundice during the acute illness. However, there were no specific clinical or physical signs uniquely characteristic during this period. Five of the 20 students required additional treatment for infections at ca. 3 mo post-IM, including acute pharyngitis in 2, non-specific diarrhea in 2 and urinary tract infection in 1. No differences could be found between males and females in clinical responses.

A regimen of adequate rest and balanced diet without alcohol intake had been stressed by the clinic personnel in an initial handout and was reinforced at each visit. Because this group's intellectual level was higher than average and good performance in school was a priority, serious effort toward establishing good dietary habits appeared in all students by the first clinic visit and was continued thereafter. However, sufficient rest was usually not achieved during the first 3–6 months.

Individual clinical variations were more prominent after 6 months. The majority of students were very aware that they were still unable to perform as well as prior to acute IM, but felt "well." In contrast, 9 of 20 at 7–8-mo- and 7 at 12-mo-post-IM continued to feel exhausted and distinctly "not well." One of these had a reoccurrence of a psychiatric illness. Four were forced by their unusual malaise and fatigue to leave school for a semester.

Total serum FA proportions post-EBV infection. In Tables 1 and 2, post-EBV average serum values of total FA present in the serum of all 20 students are compared with the values of age-matched students without EBV at monthly intervals. Proportions of the shorter-chain FA (10-13:1 and 16:1) were significantly higher ($p < 0.001$), but the essential FA, linoleic acid (18:2ω6) and the highly polyunsaturated docosahexaenoic acid (22:6ω3) were lower than normal ($p < 0.001$) during the entire year. A significant increase in the percentage of oleic acid (18:1ω9) occurred during the early post-IM period, but arachidonic acid (20:4ω6) showed phasic lowered proportions ($p < 0.001$). Figure 1 demonstrates the percentage of total FA of arachidonic acid during the year (horizontal axis) in relationship to the normal mean (solid center line) and first standard deviation of normal (dotted lines). This format is carried through subsequent figures.

Proportions of major fatty acids: Oleic acid, linoleic acid and their ratios. Figures 2 and 3 demonstrate the proportions of total FA by percentage of the major fatty acids

TABLE 1

Average Total Esterified Fatty Acid Proportions[a] in Post-EBV Serum— First 6 Months following Acute EBV Infection Compared to Normal Range

Fatty acid percent[c]	Months after EBV[b] 1	2	3	4	5	6	Normal range
10-14:1	2.7 ± 0.2*	2.1 ± 0.2*	3.2 ± 0.2*	2.8 ± 0.2*	3.2 ± 0.2*	2.7 ± 0.2*	1.4 ± 0.2
16:0	19.7 ± 2.4*	18.4 ± 1.8	18.4 ± 2.2	18.2 ± 2.4	18.8 ± 2.3	19.1 ± 1.6**	16.5 ± 2.4
16:1	3.6 ± 0.5*	2.9 ± 0.4*	3.1 ± 0.6*	3.8 ± 0.5*	3.4 ± 0.5*	3.4 ± 0.5*	2.2 ± 0.3
18:0	7.9 ± 1.5	8.1 ± 2.2	8.2 ± 1.5	7.9 ± 2.3	7.9 ± 1.5	8.2 ± 1.5	8.3 ± 1.4
18:1ω9	27.4 ± 2.0*	28.6 ± 2.3*	29.7 ± 1.9*	26.5 ± 2.1	25.7 ± 2.4	25.7 ± 2.2	24.6 ± 2.3
18:2ω6	31.8 ± 2.1*	32.2 ± 2.5*	28.3 ± 2.4*	30.3 ± 2.0*	30.8 ± 1.8*	31.1 ± 1.6*	35.4 ± 2.0
18:3ω6	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.3	0.5 ± 0.3	0.5 ± 0.3	0.4 ± 0.2	0.4 ± 0.2
18:3ω3	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
20:1ω9	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.2	0.3 ± 0.3	0.3 ± 0.2	0.2 ± 0.2
20:3ω9	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:3ω6	1.3 ± 0.3	1.3 ± 0.4	1.1 ± 0.4	1.5 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	1.5 ± 0.4
20:4ω6	6.6 ± 1.4*	7.6 ± 1.5	6.5 ± 1.1*	6.6 ± 1.2**	6.8 ± 1.5**	6.5 ± 1.5*	8.7 ± 1.5
20:5ω3	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
22:4ω6	0.7 ± 0.1**	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.2
22:5ω6	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
22:5ω3	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
22:6ω3	0.7 ± 0.2*	0.8 ± 0.2*	0.7 ± 0.2*	0.7 ± 0.2*	0.7 ± 0.2*	0.8 ± 0.2*	1.3 ± 0.2
L/O ratio[d]	1.04*	1.08*	1.01*	1.15*	1.20**	1.16*	1.4 ± 0.2
mg/ml[e]	9.6 ± 3.5	10.9 ± 3.8	14.1 ± 4.6*	13.5 ± 3.2*	12.7 ± 3.4*	12.1 ± 4.2*	8.6 ± 3.5
L + O[f]	59.2	60.8	58.1	56.8	56.5	56.8	60.0

[a]The average percentage distribution of serum FA of 20 post-IM students is compared.

[b]Comparison is at monthly intervals with the normal range of 20 age and sex-matched uninfected students during the same period.

[c]The percentage ± SD of the total FA sample.

[d]The L/O ratio is the ratio of linoleic to oleic acid.

[e]The total mg/ml serum of FA.

[f]The sum of the percentages of linoleic and oleic acids.

*Significantly different than normal, $p < 0.001$.

**Significantly different than normal, $p < 0.01$.

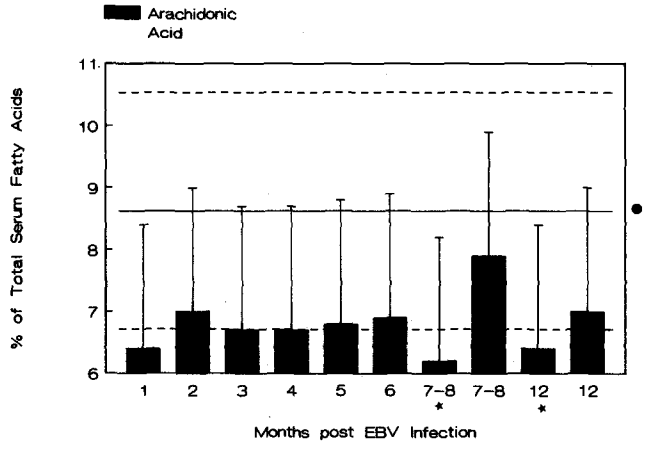

FIG. 1. Average serum proportion of arachidonic acid following acute EBV infection. The average proportion of arachidonic acid of 20 post-IM students is compared with the normal student's mean (solid line) and first SD (dotted lines) for the first 6 months after IM. At 7-8 and 12 months times, averages of students with low L/O ratios* (<1.20) are compared with averages of students with normal L/O ratios (<1.20).

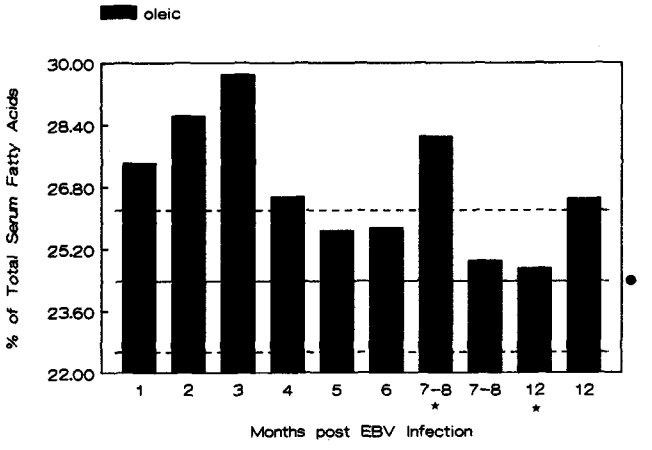

FIG. 2. Average serum proportion of oleic acid following acute EBV infection. The average proportion of oleic acid of 20 post-IM students is compared with normal mean (solid line) and first SD (dotted lines) for the first 6 months after IM. At 7-8 and 12 months, averages of students with low L/O ratios* (<1.20) are compared with averages of students with normal L/O ratios (<1.20).

TABLE 2

Average Total Esterified Fatty Acid Proportions[a] in Serum during the Second 6 Months Following Acute EBV Infection

Fatty acid percent[d]	Average 20[c]	7–8 months after EBV Low L/O 9[c]	Norm L/O 11[c]	Average 20[c]	12 months after EBV Low L/O 7[c]	Norm L/O 13[c]	0 months after EBV Normal 20[c]
10–14:1	2.0 ± 0.2	2.2 ± 0.2**	1.8 ± 0.2	3.6 ± 0.2*	3.6 ± 0.2*	3.6 ± 0.2*	1.4 ± 0.2
16:0	18.1 ± 2.4	19.4 ± 1.8*	17.5 ± 2.2	19.5 ± 2.6*	22.1 ± 2.6*	17.7 ± 1.5	16.5 ± 2.4
16:1	2.6 ± 0.6	2.9 ± 0.4*	2.4 ± 0.4	3.3 ± 0.5*	3.2 ± 0.5*	3.3 ± 0.3*	2.2 ± 0.3
18:0	8.5 ± 1.5	8.3 ± 2.2	8.8 ± 1.5	8.4 ± 1.5	7.6 ± 2.5	8.4 ± 1.5	8.3 ± 1.4
18:1ω9	26.5 ± 2.0	28.1 ± 2.3*	24.9 ± 1.9	25.5 ± 2.1	24.7 ± 2.4	26.7 ± 2.2	24.6 ± 2.3
18:2ω6	30.2 ± 2.1*	26.9 ± 2.5	33.8 ± 2.4*	31.3 ± 2.4*	28.8 ± 1.8*	33.6 ± 1.6*	35.4 ± 2.0
18:3ω6	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.3	0.5 ± 0.3	0.4 ± 0.3	0.4 ± 0.2	0.4 ± 0.2
18:3ω3	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
20:1ω9	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 0.2	0.2 ± 0.2
20:3ω9	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:3ω6	1.5 ± 0.3	1.4 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.4
20:4ω6	7.1 ± 1.5**	6.2 ± 1.5*	7.9 ± 1.5	6.7 ± 1.3**	6.4 ± 1.2**	7.0 ± 1.5**	8.7 ± 1.5
20:5ω3	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.2	0.2 ± 0.2
22:4ω6	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.2	0.2 ± 0.2
22:5ω6	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
22:5ω3	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
22:6ω3	0.9 ± 0.2**	0.8 ± 0.1*	1.1 ± 0.1**	0.9 ± 0.2*	0.7 ± 0.1*	0.9 ± 0.2**	1.3 ± 0.2
L/O ratio[e]	1.10*	0.96*	1.27	1.22**	1.14*	1.32	1.4 ± 0.1
mg/ml[f]	12.2 ± 3.4**	12.9 ± 4.1**	11.8 ± 3.2**	11.9 ± 2.9**	13.9 ± 3.9*	10.6 ± 3.5	8.6 ± 3.5
L + O[g]	56.7	55.0	58.7	57.0	53.5	60.0	60.0

[a]The average percentage distribution of serum FA of post-IM students is compared with normal range at 7–8 and at 12 months. The averages of the students with low L/O ratios (>1.20) (7–8 and 12 mo) are also compared with averages of students with normal L/O ratios (>1.20) at each time period.

[b]With separation by the linoleic/oleic acid ratios (L/O ratio) compared with normal range.

[c]Student sample number; at 7–8 months, 9 had low L/O ratios; at 12 months, 7 students had low L/O ratios.

[d]The percentage of each individual FA in the total FA ± SD.

[e]The ratio of linoleic to oleic acid; the L/O ratio.

[f]The average total serum FA mg/ml.

[g]The sum of the percentages of linoleic and oleic acids.

*Significantly different than normal, $p < 0.001$.

**Significantly different than normal, $p < 0.01$.

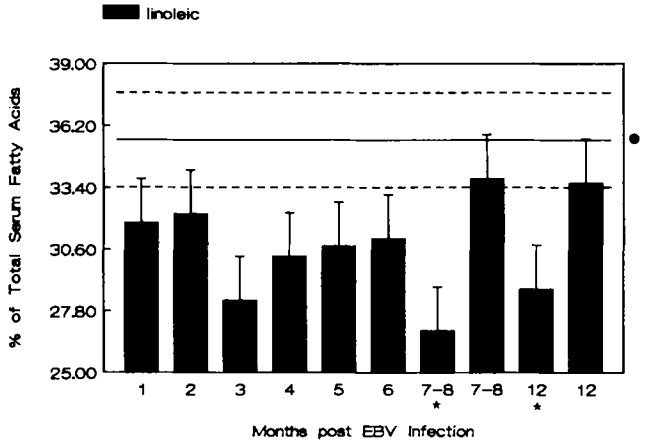

FIG. 3. Average serum proportion of linoleic acid following acute EBV infection. The average proportion of linoleic acid of 20 post-IM students is compared with normal mean (solid line) and first SD (dotted lines) for the first 6 months after IM. At 7–8 and 12 months, averages of students with low L/O ratios* (<1.20) are compared with averages of students with normal L/O ratios (<1.20).

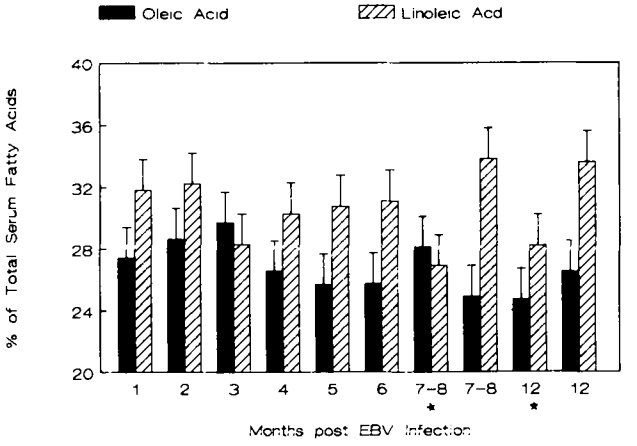

FIG. 4. Average serum proportion of oleic and linoleic acid after EBV infection. Oleic (filled) and linoleic (shaded) acid proportions in serum of post-IM students are shown together to suggest the possible reciprocal relationship between their proportions. Averages of 20 post-IM students occur for the first 6 months; at 7–8 and 12 months, averages are separated by a low L/O ratio* (<1.20) as before.

(oleic and linoleic) at monthly intervals for 1 yr after primary EBV. Marked differences from normal—increased for oleic acid (Fig. 2) and decreased for linoleic acid (Fig. 3) during the first 6 months after EBV—led us to examine their relationship by developing a ratio between them. In normal students throughout the year, the ratio of linoleic to oleic, termed the L/O ratio, was surprisingly steady at 1.44 ± 0.20. In sharp contrast, during the first 6 mo of the post-IM period, the serum L/O ratio was significantly lower than normal (Table 1, Fig. 4). In addition, although the sum of these major FA varied only slightly over the year (Tables 1 and 2), the change in their proportions was marked (Fig. 5).

We found that this ratio had returned to normal in the majority (11 of 20) of students by 6 mo, but still remained below the normal range in 9 by 7–8 mo and in 7 students at the end of the year (Table 2, Fig. 4). To evaluate whether the ratio of these major fatty acids might have a clinical significance, possibly correlating with symptoms, the clinical courses of the 7 patients with low L/O ratios at 1 yr were examined. Clinical complications during the first 6-mo-post-EBV had been experienced by 5 of these students, requiring additional treatment at the health center, and 3 of them had shown mild clinical jaundice in the acute phase of their EBV infection.

To further examine our data in the light of altered L/O ratios, the FA average proportions of the students with a L/O ratio lower than 1.20 (the first SD of normal) were separated from the remainder with L/O ratios in the normal range. Table 2 and Figures 1–3, 5 and 7–10 list these values for 9 of 20 post-IM students at 7–8 mo, and 7 of 20 post-IM students at 12 mo separately from the averages of the remaining students with normal L/O ratios.

Arachidonic acid proportion. Although the percentage of total FA of arachidonic acid (20:4ω6) was significantly lower than normal ($p < 0.005$) during the first month after EBV infection (Table 1, Fig. 1), a slight increase during the second month was followed by a second decrease during the third month ($p < 0.005$). In only 8 students was a normal proportion reached at the 6-mo period. However arachidonic acid values of clinically recovered students (normal L/O ratios) and those with low L/O ratios at 7–8 mo or at 1 yr after acute EBV were not very different. These alterations suggest the possibility of several metabolic influences on serum arachidonic acid proportions (Table 2).

Fatty acid elongation and desaturation enzyme pathways. Figure 6 demonstrates FA pathways of elongation and desaturation from the essential FA. Elongation is carried out at the COOH moiety by mitochondrial acetyl CoA enzyme, and desaturation is accomplished at specific C positions by specific cytochrome b5 oxygenases with NADPH under configurational restraints (7,11–13). Essential FA, linoleic (18:2ω6) and α-linolenic (18:3ω3) acids, and also oleic acid are initially desaturated by the key enzyme Δ6-desaturase. Elongation, normally a reversible process occurring between desaturation steps, can operate in excess when desaturase enzymes are unavailable due to overuse or lack of function (11–13). As indicated in Figure 6, Δ5-desaturase forms 20:5ω3 (eicosapentaenoic acid) from 20:4ω3, and 20:4ω6 (arachidonic acid) from 20:3ω6. Δ4-Desaturase accomplishes the final hydrogen removal from 22:4ω6 and 22:5ω3 to form 22:5ω6 and 22:6ω3.

Estimated desaturation enzymes activities. Figures 7–9 compare the average estimated post-EBV activities of three desaturase enzymes with average estimated normal activity (central line), according to the calculations of Holman and Johnson (15). The Δ6-desaturase nadir at 3 mo post-infection corresponds to the maximal alterations found in FA profiles (Fig. 7). Low values of both Δ5- (Fig. 8) and Δ4- desaturases (Fig. 9) may reflect lack of substrates due to apparent defective activity of Δ6-desaturase enzyme, because these desaturase enzymes are further steps in the same metabolic pathways. For comparison, students with L/O ratios of lower than 1.2 (as indicated by asterisk) after 6 mo are shown by separated average bars at the 7–8- and 12-mo periods (Figs. 7–9).

Estimated elongation enzymes activities. Elongation enzyme activities, estimated by Holman and Johnson Metabolic Indices (15), are compared with estimated normal activity in Figures 10 and 11. Although essential α-linoleic acid (18:3ω3) is slightly elevated during the first 6 mo (Table 1), in contrast, all ω3 elongation products are

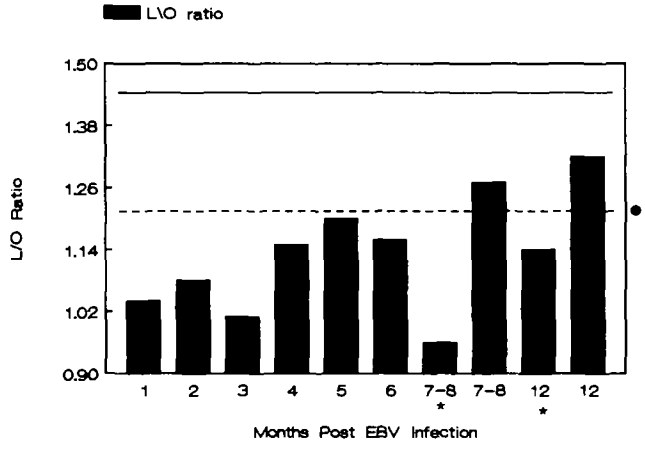

FIG. 5. Linoleic to oleic acid ratio; L/O ratio, following acute EBV infection. The L/O Ratio, determined by dividing the percentage of linoleic acid in the total serum FA by the oleic acid percentage, after acute IM is compared with the normal and mean and first SD. Averages of 20 post-IM students are shown from 1–6 months, and at 7–8 and 12 months, students with low L/O ratios* (<1.20) are shown separately from other post-IM students with normal L/O ratios.

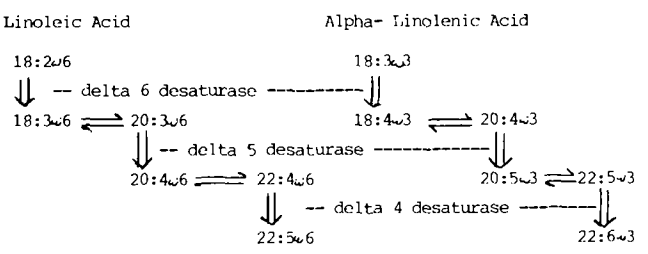

FIG. 6. Desaturation and elongation pathways of the essential fatty acids: Linoleic and α-linolenic acids. The metabolic pathways of the essential fatty acids, linoleic (ω6 series) and α-linolenic (ω3 series) are depicted schematically, with desaturation shown vertically and elongation horizontally. Specific FA configurations serving as substrates for individual desaturase enzymes are shown by dotted lines.

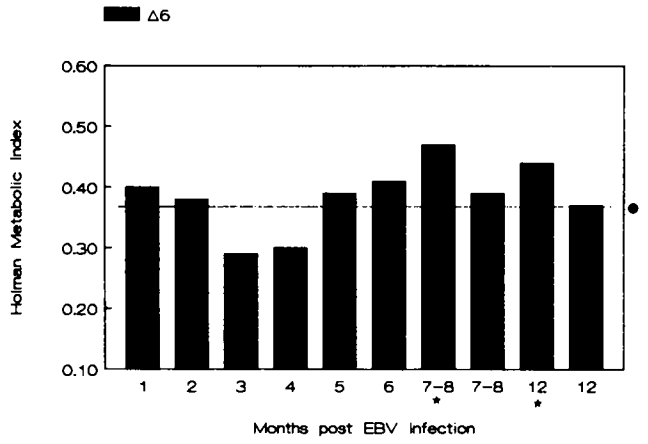

FIG. 7. Estimated Δ6-desaturase activity after EBV infection. Metabolic indices of Holman and Johnson (15) have been calculated from individual FA averaged percentage at each month for 1 year to estimate the metabolic activity of Δ6-desaturase. Averages of 20 students from 1–6 months post-IM, and averages of students separated by L/O ratio are compared with the normal mean (solid line). Low L/O ratio (<1.20) averages are noted by an asterisk (*).

lower than normal during that time (Fig. 10), possibly because their precursor, 18:3ω3, is not available as a suitable substrate for further action. Estimated activities of C20 and C22 elongation enzymes (Fig. 11), however, suggest they are functioning in excess intermittently, possibly storing elongated FA until desaturation processes can normalize pathway flow (Fig. 6). A representative GLC FA chromatograph of a 3-months-post-IM patient's total FA profile shows accumulations of unusual peaks (marked by arrows on Fig. 12) at the expected retention times of "elongation stop-point products" (12,13). Similar unusual chromatograph peaks were commonly found in the FA profiles during the first 3 mo after EBV infection, but were usually absent from later serum samples.

EBV-specific serum antibodies. One-year-post-EBV infection serum samples from each of 7 post-IM students with persistent clinical symptoms and altered linoleic/oleic acid ratios (<1.20) were negative for EBV-IgA and EBV-early-antigen-IgG antibodies.

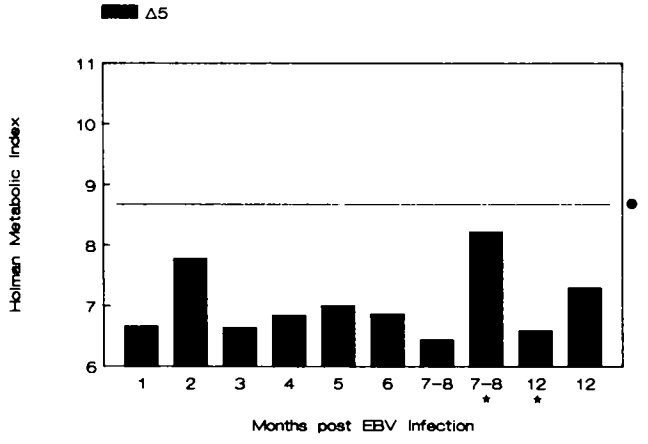

FIG. 8. Estimated Δ5-desaturase activity after EBV infection. Format as described in Figure 7 with Δ5-desaturase enzyme activity estimated by metabolic index calculations (15).

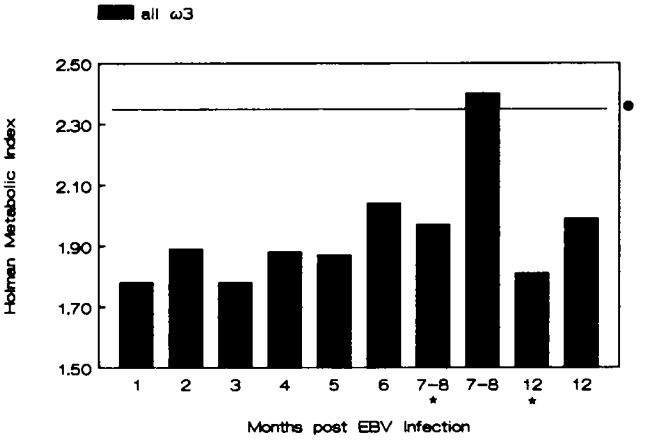

FIG. 10. Estimated ω3 elongation enzyme activities after EBV infection. Format as described in Figure 7 with all ω3 elongation enzyme activities estimated by metabolic index calculations (15).

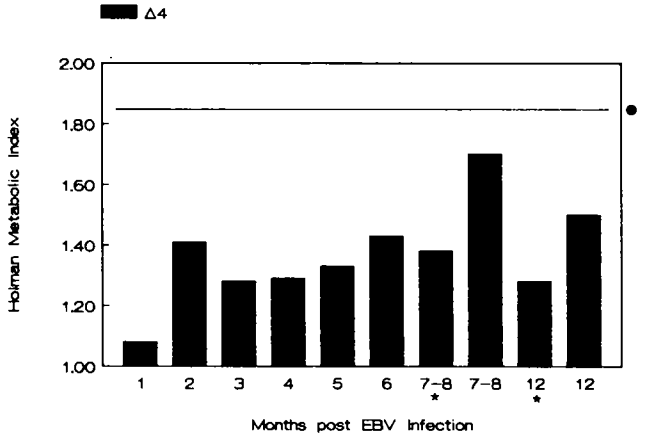

FIG. 9. Estimated δ4-desaturase activity after EBV infection. Format as described in Figure 7 with Δ4-desaturase enzyme activity estimated by metabolic index calculations (15).

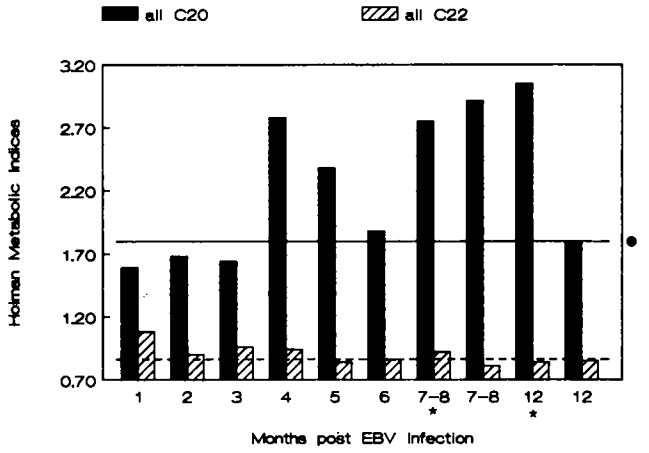

FIG. 11. Estimated C20 and C22 elongation enzyme activities after EBV infection. Format as described in Figure 7, with C20 and C22 elongation enzyme activities estimated by metabolic index calculations (15).

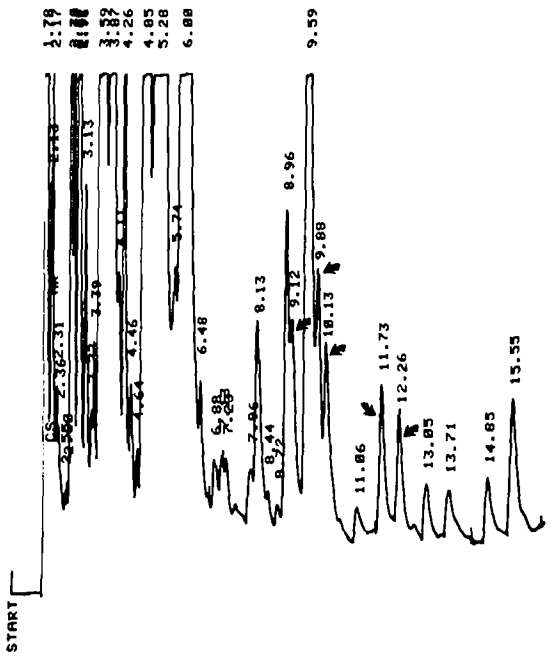

FIG. 12. Gas liquid chromatograph record of total fatty acid proportions of a post-IM student at 3 months after acute EBV infection. Methyl esters of fatty acids, visualized by flame-ionization technique are shown in the sequence of their carbon length, beginning with the shortest (10:0–12) at 2.13 min, and continuing to the longest, 22:6ω3 at 15.55 min. Altered proportions of particular note are oleic acid (5.28 min) = 21.55% of total FA; linoleic (6.0 min) = 23.4%, or 64.8% of the normal mean; arachidonic acid (9.59 min) = 4.54%, or 52.3% of the normal mean; and docosahexaenoic acid (15.55 min) = 0.66% of the total FA, or 53% of the normal mean. The L/O ratio is 1.09, significantly below normal ($p < 0.001$). "Unusual" chromatography peaks marked by arrows occur at C20 (9.12, 9.88 and 10.13 min) and C22 (11.73 and 12.26 min) elongation product positions. These peaks were absent the following month in this student's serum.

DISCUSSION

Alterations in FA composition, measured during months after acute EBV infection—IM—suggest that changes in host lipid metabolism may persist after the primary illness has apparently terminated. By beginning after clinical recovery, our study plan bypassed the known confounding effects of acute illness on serum FA that may be due to inadequate diet, fever, prostration and tissue necrosis (2-6). FA serum profiles of normal college students during the entire year after apparent remission from acute IM showed that serum proportions of the major serum FA, oleic, linoleic, palmitic and arachidonic acids are markedly affected. The following common pattern emerged: Initial values obtained at 1-mo-post acute IM reflected the severe effects common to a primary infection. Then, during the second mo-post-IM, FA profiles appeared to be returning to normal. However, surprisingly, the most significant alterations of FA profiles occurred at 3-4-mo-post-IM, when clinical recovery appeared underway. A slow recovery of FA ratios toward normal usually followed during the subsequent 6 mo. A lack of differences among male and female responses in either biochemical or clinical findings suggested that the severity of the EBV-disease process may have obscured this possibility (7).

Individual patient variations in this pattern, particularly a lower than normal linoleic to oleic acid ratio (<1.20), led us to evaluate individual clinical courses. It appeared that the 7 of 20 students whose FA values had not returned to normal at 1 yr had also experienced more fatigue and difficulty in returning to full activity than the remaining 13 students. Secondary infections that required treatment in 5 of those 7 students shortly after acute IM may have possibly contributed to their slower recovery. Alternatively, a low L/O ratio prior to IM may have led to the development of disease susceptibility. However, none of these students retained IgG or IgA antibody titers to early EBV antigens at 1-yr-post-IM that might suggest a chronic EBV state (16).

To test for a possible clinical association, we separated the FA profiles of the 9 students who had retained low L/O ratios at 7-8 mo and the 7 with low L/O ratios at 1 yr. This selection clearly identified students who expressed more "fatigue" than those with normal L/O ratios, and who had altered their life pattern because of their continuing "malaise." To examine this effect on their entire FA profiles, average FA proportions of students with normal L/O ratios were separated from averages of those with lowered L/O ratios (Table 2, Figs. 1-3 and 7-11). We found that abnormal proportions of other FA and altered FA enzyme activities were also detected in the low L/O ratio group when compared with those having normal L/O ratios post-IM and normals without EBV. We could conclude from these clinical and biochemical correlations that the L/O ratio might be effective as a predictive marker for slow recovery from acute EBV infection, or possibly in other chronic illnesses (2-6). Superimposed infection in the post-IM period, found in several of thes students, also may have prolonged recovery by causing an additional burden on FA metabolism (4,5).

Oleic acid, normally 25.4% and linoleic acid, 35.6%, comprise approximately 60% of serum total-lipoprotein FA (7). Hepatic microsomes are the metabolic source of the large quantities of these FA necessary to sustain normal serum-lipoprotein content. Therefore, major alterations in total serum-FA composition suggest the possibility that liver may continue to be a site of host-viral interaction even after the acute viral hepatic phase. Despite the return to normal values found in the usual panel of liver enzymes after acute IM (1,17,18) and the absence of jaundice, hepatic enlargement or abdominal tenderness in the students of our study, an EBV-related intrahepatic cellular abnormality of FA enzyme activity appears present for at least 4 mo after EBV infection. However, metabolic turnover studies in liver tissue would be necessary to resolve this possibility. EBV latency, known to occur in a subset of B lymphocytes and epithelial tissues, has not been reported in hepatic tissue (20). Although a reciprocal relationship has not been proposed between these FA, it is interesting that the contribution of the total portion of the FA profile by linoleic and oleic acids together did not change appreciably during the year despite marked variations in their individual proportions (Tables 1 and 2; Fig. 5).

We estimated the activities of FA elongation and desaturation enzymes post-IM (15). Because specificity of individual FA enzymes for a particular FA double-bond configuration limits its utilization, specific sequential processing of individual FA can be followed (Fig. 5) (11-13,15). Enzyme activities of elongation and desaturation can be estimated from their proportions. Comparison

of values with normals allows evaluation of FA metabolism in disease states (15). Post-EBV FA elongation enzymes appeared to be functioning normally or in excess after EBV infection (Figs. 6,7). In contrast, activities of the Δ6-, Δ5- and Δ4-desaturases appeared lower than normal, particularly at 3-mo-post infection (Figs. 8–10). The possibility of low FA-desaturase activity was supported by the frequent finding of unusual accumulations of FA at the retention times consistent with C20 and C22 elongated FA compounds (Fig. 11) (13). It is known that, during low activity of desaturase enzymes, reversibly elongated "stop-point" FA can be held temporarily until further enzyme availability (13). Eventually, after reversal of elongation or truncating of the carbon chain, the "stop-point" FA reenter the metabolic pool for further desaturation steps (12,13). Although these FA occur in minute amounts (~1%) in normal blood samples, the majority of post-EBV patients showed noticeable accumulation of apparent "stop-point" FA at some time during the first 2–4 mo, at the time when estimated FA-desaturation activity appeared low (Fig. 11).

Lower serum proportions of other FA may be of clinical significance following acute EBV infection. Low total arachidonic acid, as the FA precursor of immunoregulatory metabolites, has been associated with increased amounts of prostaglandin and leukotriene formation (10,20). Although increases in these immunoregulatory compounds have been measured during the acute phase of EBV-illness (21), they have not been evaluated during the longer postinfection period examined here. In addition, because our method also extracts shorter-chain FA (2,20), the significantly high proportions of 10:0–14:1 FA may represent breakdown products of longer-chain FA (10). Another long-chain polyunsaturate, docosahexaenoic acid (22:6ω3), was present in less than normal proportion in serum after EBV infection. A possible deficit in supply of this FA for red-muscle metabolism, specifically the slow phase of muscle contraction (7), may be a factor in the common post-EBV complaint of "weakness." Moreover, rare neurological complications following EBV infection such as Guillain-Barré and Horner's syndromes or deafness (22,23) may have been augmented by a possible lack of 22:6ω3, an essential neuronal substrate (10). Low serum-lipoprotein arachidonic and docosahexaenoic acid proportions suggest that these FA stores are being rapidly depleted by specific metabolic needs (21), that they are being formed poorly, or both. Our data cannot distinguish among these possibilities.

Although clearly not specific for EBV infection (2–6,20), an abnormally low ratio of serum linoleic to oleic acid appears after clinical recovery in IM. Abnormal proportions of these major FA appear to be associated with increased clinical symptoms in some post-IM patients and other chronic diseases (2,3,20). Abnormality in this FA ratio may reflect a transient but crucial phase of recovery post-IM. Whether this marker can be used as a diagnostic tool in evaluating the EBV-recovery process remains to be determined.

ACKNOWLEDGMENT

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REFERENCES

1. Rubenstein, E. and Federman, D.D., eds. (1985) in *Scientific American Medicine*, Vol. 2, Infectious Diseases XXVI, pp. 7–9, Scientific American Inc., New York, NY.
2. Love, C.W., Chashell, A., Reynolds, M., and Callaghan, N. (1974) *Br. Med. J. 3*, 18–21.
3. Mertin, J., and Meade, C.J. (1977) *Br. Med. Bull. 33*, 67–71.
4. Campbell, I.M., Crozier, D.N., and Caton, R.B. (1976) *Pediatrics 57*, 480–486.
5. Miura, S., Asakura, H., Morishita, T., Kobayashi, K., Yoshioka, M., Morita, A., and Tsuchiya, M. (1982) *Digestion 25*, 103–114.
6. Mamunes, P., DeVries, G.H., Miller, C.D., and David, R.B. (1974) in *Reye's Syndrome* (Pollack, J.D., ed.) pp. 245–254, Grune & Stratton, New York, NY.
7. Lehninger, A.L. (1975) in *Biochemistry*, 2nd ed., Chap. 11, Lipids, Worth Publishing, Inc., New York, NY.
8. Lotz, M., Tsoukas, C.D., Carson, D.A., and Vaughn, J.H. (1987) in *Epstein-Barr Virus And Human Disease* (Levine, P.H., Ablashi, D.V., Nonoyama, M., Pearson, G.R., Glaser, R., eds.) pp. 349–353, Humana Press, Clifton, NJ.
9. Spector, A.A., and Yorek, M.A. (1985) *J. Lipid Res. 26*, 1015–1035.
10. Birkle, D.L., and Bazan, N.G. (1986) in *Progress in Retinal Research* (Osborne, N., and Chader, J., eds.) Vol. 5, pp. 309–335, Pergamon Press, Oxford, England.
11. Neufeld, E.J., Sprecher, H., Evans, R.W., and Majerus, P.W. (1984) *J. Lipid Res. 25*, 288–293.
12. James, A.T. (1977) *Adv. Exp. Med. Biol. 83*, 51–73.
13. Brenner, R.R. (1977) *Adv. Exp. Med. Biol. 83*, 85–101.
14. Snedecor, G.W., and Cochron, W.G. (1980) *Statistical Methods*, 7th edn., Iowa University Press, Ames, IA.
15. Holman, R.T., and Johnson, S.B. (1983) *Dietary Fats and Health* (Perkins, E.G., and Visek, W.J., eds.) pp. 246–266, American Oil Chemists' Society, Champaign, IL.
16. Beisel, W.R., and Fiser, R.H. Jr. (1970) *Am. J. Clin. Nutr. 23*, 1069–1079.
17. Straus, S.E., Tosato, G., Armstrong, G., Lawley, T., Preble, O.T., Henle, W., Davey, R., Pearson, G., Epstein, J., Brus, I., and Blaese, R.M. (1985) *Ann. Intern. Med. 102*, 7–16.
18. Jones, J.F., Ray, G., Minnich, L.L., Hicks, M.J., Kibler, R., and Lucas, D.O. (1985) *Ann. Intern. Med. 102*, 1–7.
19. Rickinson, A.B., Moss, D.J., Pope, J.H., and Ahlberg, F. (1980) *Int. J. Cancer 25*, 59–65.
20. Williams, L.L., O'Dougherty, M.M., Wright, F.S., Bobulski, R.J., and Horrocks, L.A. (1986) *Neurology 36*, 1200–1205.
21. Pagano, J.S., and Nedrud, J.G. (1980) in *The Human Herpesviruses* (Nahmias, A.J., Dowdle, W.R., and Schinazi, R.E., eds.) pp. 206–220, Elsevier Press, New York, NY.
22. Grose, C., Henle, W., Henle, G., and Feorino, P.M. (1975) *N. Engl. J. Med. 292*, 392–395.
23. Williams, L.L., Lowery, H.W., and Glaser, R. (1975) *Pediatrics 75*, 1020–1027.

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METHODS

Thiophosphoester Analogs of Phosphatidic Acids: Spectrophotometric Substrates for Phosphomonoesterases[1]

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Thiophosphoester analogs of dioctanoyl and didecanoyl phosphatidic acids were synthesized for use as substrates in spectrophotometric assays. These substrates are easily dispersable in aqueous media and release thiodiacylglycerols after phosphomonoesterase catalyzed hydrolysis. The free sulfhydryl of these thiodiacylglycerols reacts with the colorimetric reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), allowing the reaction to be followed. These analogs were shown to be good substrates for calf intestine alkaline phosphatase (highest activity at alkaline pH) and phosphomonoesterases of partially purified beef brain cytosol (highest activity at physiologic pH). Cationic amphiphilic drugs inhibit the actions of alkaline phosphatase on the dioctanoyl analog, but did not inhibit enzymatic hydrolysis of *p*-nitrophenyl phosphate. In contrast, the beef brain cytosolic fraction *p*-nitrophenyl phosphate hydrolysis was mildly inhibited, and the phosphatidic acid analog hydrolysis was increased slightly. Tetramisole inhibited all enzyme activities with *p*-nitrophenyl phosphate, but was inhibitory only to the alkaline-phosphatase activity with the phosphatidic acid analog. *Lipids 23,* 989–992 (1988).

Alkaline phosphatase (EC 3.1.3.1) catalyzes the hydrolysis of many water soluble substrates (1) and of the phospholipid phosphatidic acid (PA) (2,3). Phosphatidic acid phosphohydrolase (EC 3.1.3.4) catalyzed hydrolysis of PA is thought to be distinct and to differ from alkaline-phosphatase activity on the basis of substrate specificity, pH requirements and enzyme response to various ions (4). Phosphatidic acid phosphohydrolase activity is usually determined by substrate radiolabeling or orthophosphate release (4). Some common radiolabeled substrates, or substrate precursors, are [^{32}P]PA, [^{32}P]ATP and a novel PA analog, 1-*O*-hexadecyl-*rac*-[2-^{3}H]glycerol-3-phosphate (4). The thiophosphate PA analog presented here has potential for use in determining the activity of various phosphomonoesterases, including alkaline phosphatase and phosphatidic acid phosphohydrolase, in crude and purified preparations.

MATERIALS AND METHODS

Synthesis of thiodiacylglycerols. The method of Snyder (5) was used to synthesize dioctanoyl and didecanoyl thiodiacylglycerols from octanoyl and decanoyl chlorides (Aldrich, Milwaukee, WI), respectively. Products were further purified by thin layer chromatography (TLC).

Synthesis of thiophosphatidic acids. Dioctanoyl and didecanoyl thiodiacylglycerols were reacted in dry pyridine with methyl dichlorophosphate (Aldrich), by a modification of the method of Smrt and Catlin (6), to form dioctanoyl and didecanoyl thiophosphatidic acids (S-PA-8 and S-PA-10). Three hundred mg of thiodiacylglycerol were dissolved in 3 ml of dry pyridine. In a separate 100 ml three-necked round bottom flask, fitted with a $CaCl_2$ drying tube and rubber septum, were placed 8 ml of dry pyridine. A pasteur pipette was run through a rubber septum into the flask so that the tip reached into the reaction mixture. A flask was equipped with a magnetic stirrer and a stream of nitrogen was bubbled through the mixture. One ml of methyl dichlorophosphate was added, with a 2 ml glass syringe through the septum, while stirring. The thiodiacylglycerol solution was added dropwise 10 min later. After 3 hr, no thiodiacylglycerol was detected by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) spray on thin layer chromatograms. The white precipitates were suction filtered, followed by addition of 3 ml of 0.2 M Na_2HPO_4/NaH_2PO_4, pH 8.4 buffer, for 15 min with magnetic stirring. The pyridinium salts were extracted with $CHCl_3$ and the lower phase rotary evaporated at 47°C to dryness. Residues were dissolved in $CHCl_3$ and passed through Whatman PS-1 paper. After further rotary evaporation, the solids were dissolved in a small amount of $CHCl_3$ and purified by silica gel (Unisil, 100–200 mesh) column chromatography. S-PA comigrated with dipalmitoyl PA (Sigma, St. Louis, MO) on silica gel TLC plates at R_f 0.10, solvent system $CHCl_3/CH_3OH/NH_4OH$ (60:35:5, v/v/v). Phospholipids were detected by iodine vapor and a phospholipid spray (7). S-PA also were identified by ^{31}P and ^{1}H NMR (IBM WP-200SY 200 MHz FT-NMR), IR (Sargent Welch 3-200 IR spectrophotometer), and C, H, N elemental analysis (Perkin-Elmer Elemental Analyzer). Lipid phosphorus was determined by the method of Ames (8). S-PA released thiodiacylglycerols after enzymatic hydrolysis with alkaline phosphatase as indicated by TLC with DTNB spray (7) and comigration with starting thiodiacylglycerols. IR (film on NaCl) 2970 and 2860 (broad; C–H stretching), 1740 (sharp, strong; ester bonds), 1480 and 1390 (broad; C–H bonds), 1100–1250 (broad; ester bonds), 750 and 687 (sharp, weak; PO_2^-), 1540 and 1630 (sharp, weak; pyridinium). ^{1}H NMR (tetramethylsilane, ppm) 0.9 (terminal-CH_3), 1.3 (-CH_2-chains), 1.6 (CH_2-C-CO), 2.3 (triplett; CH_2-CO), 2.9, 3.0, 5.3 (C–H of glycerol backbone), 4.1 and 4.4 (C-CH-S-), 7.8, 8.2, 8.9 (pyridine ring), 12.2 (H-N^+). ^{31}P NMR (phosphoric acid standard, ppm) dipalmitoyl PA, 1.88, S-PA, 23.50. Elemental analysis (S-PA-10, pyridinium salt) ($C_{28}H_{50}O_7NPS$)—calculated: C, 56.83; H, 8.52; N, 2.37; P, 5.32; and found, C, 56.75; H, 8.62; N, 2.28; P, 5.37.

[1]This research was completed as partial fulfillment of the Ph.D. degree, awarded August, 1987, at Northern Illinois University, DeKalb, IL.

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PA, phosphatidic acid; S-PA-8, *rac*-1-phospho-2,3-*O*-dioctanoyl-1-mercapto-2,3-propanediol; S-PA-10, *rac*-1-phospho-2,3-*O*-didecanoyl-1-mercapto-2,3-propanediol; TLC, thin layer chromatography.

Alkaline phosphatase catalyzed hydrolysis. Immediately before use, substrates were prepared from stock solutions by silica gel TLC (Sigma, 250 μm glass plates). Residues were removed from the silica gel with $CHCl_3$, rotary evaporated and further dried by a stream of N_2 gas. Twenty ml of 10 mM Tris-HCl, pH 7.7, were added, followed by 3 min of rapid stirring with a Biospec handheld high-speed homogenizer set at 10,800 rpm. This treatment gave optically clear and stable solutions. Assays were carried out in 1 × 1 cm quartz cuvettes with 9.6 μM DTNB, 8.8 μM substrate and varying amounts of calf intestine alkaline phosphatase (Sigma, Type VII, P5521). A typical assay proceeded first by mixing the buffer and substrate, then adding the enzyme. After 10 rapid strokes with a Teflon strip, the DTNB (in 10 μl) was added and stirring repeated. Because of substrate self-hydrolysis, cuvettes without alkaline phosphatase were treated in a similar manner as above and these values subtracted from rates. This autolysis rate ranged from about 50% of enzymatic rate, for low concentrations of protein, to 5% with higher concentrations. Reactions were monitored with a Spectronic 501 single-beam spectrophotometer (Bausch and Lomb, Rochester, NY) at 412 nm wavelength. All absorbances were transformed to nmol using the molar extinction coefficient of 12,800 (5). Three determinations of each point were obtained and the means of each was used to determine linear regressions.

Beef brain cytosol enzymatic hydrolysis. A partially purified cytosolic preparation from beef brain was prepared similarly to the method of Berglund et al. (9), for rat liver phosphatidic acid phosphohydrolase preparation. Assay conditions were similar to that for alkaline-phosphatase reactions, except the final concentration of DTNB was 17 μM and 40 μM in the substrate. The protein concentration was estimated by the method of Lowry et al. (10).

pH profiles of enzymatic reactions. S-PA-10 was tested over a range of pH's with alkaline phosphatase and beef brain cytosol enzymes. Conditions were those described in the assay sections with the exception of the buffers. Three buffers were used to cover a 5.5–9.0 range of pH's. For 5.5–7.0, 4-morpholineethanesulfonic acid was used. For pH 6.5–8.0, 4-morpholinepropanesulfonic acid was used. For the pH range of 7.5–9.0 , (3-[tris(hydroxymethyl)-methylamino]-1-propanesulfonic) was used. All buffers had a final concentration of 50 mM. Fifty μg of protein was used per cytosolic assay and 32 ng in alkaline phosphatase assays. Each data point was the mean of 3 determinations (6 for 6.5–8.0, because of buffer overlap).

Effects of tetramisole and cationic amphiphilic drugs on alkaline phosphatase activity. A 20 ml dispersion of S-PA-10 was prepared in 10 mM Tris-HCl, pH 7.6, by sonicating the substrate for 45 sec with a Sonic Dismembrator (Artek Systems, Farmingdale, NY) at the 45% setting, using a 1/2 in. diameter horn. Triton X-100 (Sigma) was then added to give a final concentration of 0.1% (v/v). Assay conditions were similar to those presented above, except final concentrations were 21 μM substrate and 48 μM DTNB. Chlorpromazine, DL-propranolol, tetramisole (+/−) and tetracaine (Sigma) were added to each cuvette before alkaline phosphatase addition.

Alkaline phosphatase was also tested in the presence of these drugs with *p*-nitrophenyl phosphate (Calbiochem, La Jolla, CA). Final concentrations in quartz cuvettes were: 50 μM *p*-nitrophenyl phosphate, 1 mM $MgCl_2$, 0.01% Triton X-100 and 11 ng/ml alkaline phosphatase in 10 mM Tris-HCl, pH 7.6, in a total volume of 3 ml. Assays were similar to those for the thiophosphate analogs, except hydrolysis was followed at 410 nm wavelength and DTNB was not used. Concentrations of cationic amphiphilic drugs were: 0.5 mM chlorpromazine, 10 mM tetramisole, 5 mM propranolol, and 5 mM tetracaine.

Beef brain cytosol was tested with these drugs, although chlorpromazine could not be used under these conditions, because of turbidity after addition to partially purified cytosol. Conditions were similar to those for alkaline phosphatase and the linearity studies described already.

RESULTS AND DISCUSSION

These thiophosphate phosphatidic acid analogs are good substrates for calf intestine alkaline phosphatase and beef brain phosphomonoesterases (Figs. 1 and 2). Although evidence exists for alkaline-phosphatase catalyzed hydrolysis of nonlipid thiophosphomonoesterase (1) and phospholipase hydrolysis of thiol-releasing substrates that use DTNB for product quantitation (5,11), analogous thiophosphatidic acid hydrolysis is reported here for the first time. Calf intestine alkaline phosphatase also has been show to stimulate hydrolysis of egg-derived PA in a highly specific manner (2). The data presented here show an alkaline phosphatase specificity toward *p*-nitrophenyl phosphate over S-PA (Table 1). The solution pH requirement for alkaline phosphatase actions on S-PA follows a classical alkaline phosphatase pH curve for nonlipid substrates such as *p*-nitrophenyl phosphate (Fig. 3). Beef brain cytosolic enzymes appear to contain phosphomonoesterases, which are most active at physiologic pH's (Fig. 3). This could be a reflection of brain phosphatidic acid phosphohydrolase, which has been reported to have pH optima in this range (12).

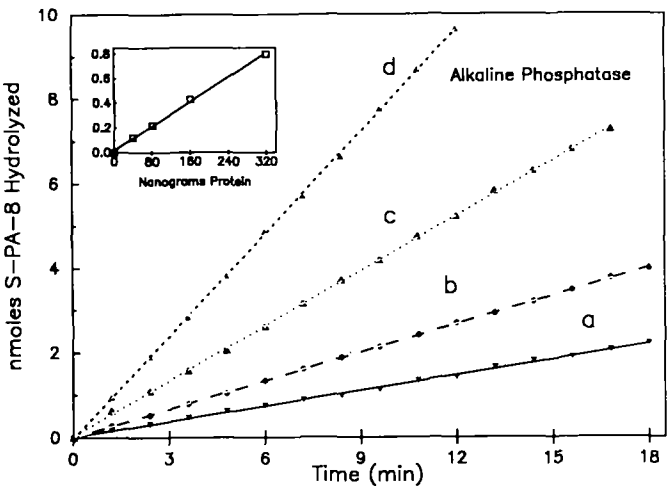

FIG. 1. Alkaline phosphatase catalyzed hydrolysis of S-PA-8 analog. Conditions as described in Methods. a–d, ng Protein in assays: a (40), b (80), c (160), d (320). Inset shows linear relationship of rate of hydrolysis with protein concentration. Correlation coefficients are >0.985.

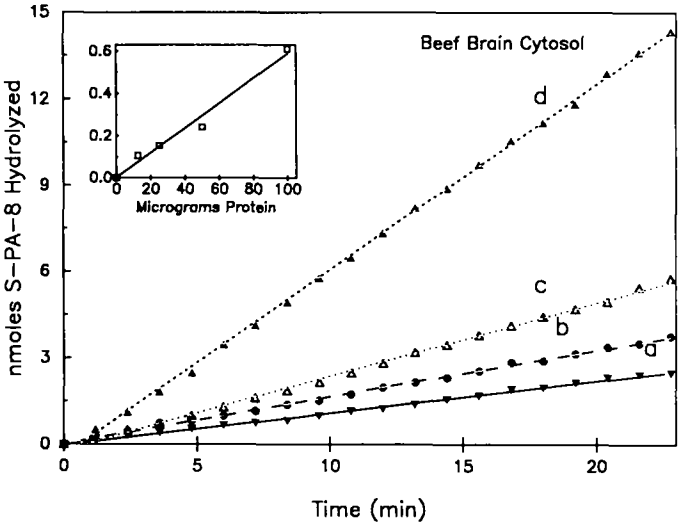

FIG. 2. Beef brain cytosol catalyzed hydrolysis of S-PA-8 analog. Conditions as described in Methods. a–d, μg Protein in assays: a (12.5), b (25), c (50), d (100). Inset shows linear relationship of rate of hydrolysis with protein concentration. Correlation coefficients are >0.985.

TABLE 1

Substrate Specificity

	p-Nitrophenyl phosphate	S-PA-8
Alkaline phosphatase[a]	23.69	2.78
Beef brain cytosol[b]	4.20	5.51

[a]nmol hydrolyzed/μg protein/min.

[b]nmol hydrolyzed/mg protein/min.

Conditions as described in Methods.

All correlation coefficients >0.985, using the means of three determinations for each point of the linear regression.

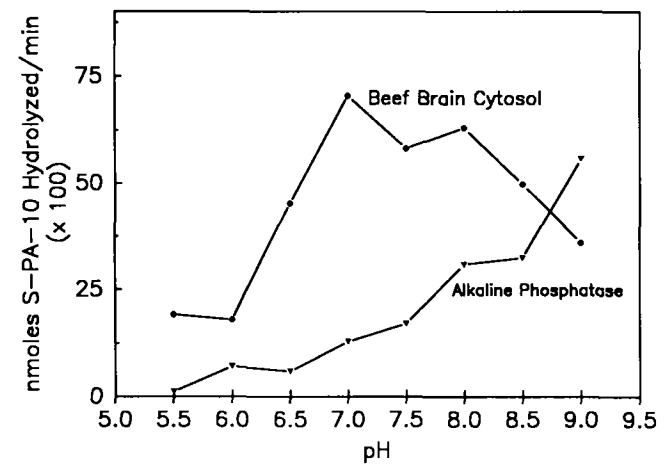

FIG. 3. pH profile of alkaline phosphatase and beef brain cytosol catalyzed hydrolysis of S-PA-10 substrate. Conditions as described in Methods. All correlation coefficients of linear regressions used to plot points are >0.980.

When examining the effects of cationic amphiphilic drugs on alkaline phosphatase catalyzed hydrolysis, substrate types (lipid vs nonlipid) were differentially affected (Tables 2 and 3). Tetracaine, propranolol and chlorpromazine strongly inhibited the enzyme activity towards S-PA-8. With *p*-nitrophenyl phosphate substrate, tetracaine and chlorpromazine had no effect on hydrolytic rate, but propranolol gave a 15% increase. Tetramisole (+/−), a very potent alkaline phosphatase inhibitor (1), inhibited all reactions with both substrates, except with S-PA-8 in brain cytosol (Table 3). Although phosphatidic acid-phosphohydrolase activity was not further purified, the activity towards S-PA-8 in beef brain cytosol is most likely due to this enzyme and not alkaline phosphatase, as seen by the effects of amphiphilic amines and tetramisole. If the activity were to be attributed to alkaline phosphatase, dramatic decreases would be expected, when in fact, the activity is almost unchanged (slight increases). The contrast with tetramisole is most dramatic, because it is a well-defined inhibitor of alkaline phosphatase (1). Cationic amphiphilic drugs have been reported to inhibit phosphatidic acid phosphohydrolase activity with PA substrates (13), and this is seen to be the case with alkaline phosphatase and S-PA. This inhibition is thought to be the result of the amphiphilic amine's ability to prevent the relatively inactive cytosolic phosphatidic acid phosphohydrolase from interacting with microsomal membranes, where activity is much higher (14).

TABLE 2

Actions of Amphiphilic Amines and Tetramisole on Alkaline Phosphatase

	Substrate	
	S-PA-8	*p*-Nitrophenyl phosphate
Tetramisole	−41	−33
Tetracaine	−85	0
Chlorpromazine	−100	0
Propranolol	−96	+15

Values are percentage of the rate of hydrolysis without added compound.

Conditions described in methods.

TABLE 3

Actions of Amphiphilic Amines and Tetramisole on Beef Brain Enzymes

	Substrate	
	S-PA-8	*p*-Nitrophenyl phosphate
Tetramisole	+8	−25
Tetracaine	+15	−13
Propranolol	+16	−13

Values are percentage of the rate of hydrolysis without added compound.

Conditions described in Methods.

The slight stimulation seen in the cytosol is consistent with Koul and Hauser (15) and Martin et al. (16), who reported similar increases with chlorpromazine. It is interesting to note that Koul and Hauser (15) found that sonicating PA preparations caused an increase, but emulsions as substrate preparation lead to amphiphilic amine-induced decreases in activity. The thiophosphate PA is readily dispersable by sonication or high-speed stirring and prepared, by this method, for assays.

In conclusion, these analogs are readily and economically synthesized, excellent substrates for phosphomonoesterases, and are more sensitive to enzymes requiring physiological pH's. They are poor alkaline phosphatase substrates in the presence of cationic amphiphilic drugs. Partially purified beef brain cytosolic fraction is apparently weak in alkaline phosphatase activity, and hydrolysis of S-PA-8 is probably a result of phosphatidic acid-phosphohydrolase activity.

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REFERENCES

1. McComb, R.B., Bowers, G.N. Jr., and Posen, S. (1979) in *Alkaline Phosphatase,* pp. 229-277, Plenum Press, New York, NY.
2. Ehle, H., Muller, E., and Horn, A. (1985) *FEBS Lett. 183,* 413-416.
3. Sumikawa, K., Saeki, K., Okochi, T., Adachi, K., and Nishimura, H. (1987) *Clin. Chim. Acta 167,* 321-328.
4. Bleasdale, J.E., and Johnston, J.M. (1982) in *Lung Development: Biological and Clinical Perspectives* (Farrell, P.M., ed.), Vol. 1, pp. 269-294, Academic Press, New York, NY.
5. Snyder, W.R. (1987) *J. Lipid Res. 28,* 949-954.
6. Smrt, J., and Catlin, J. (1970) *Tetrahedron Lett. 58,* 5081-5082.
7. Kates, M. (1972) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T.S. and Work, E., eds.) Vol. 3, pp. 436-437, North-Holland, Amsterdam, The Netherlands.
8. Ames, B.N. (1966) in *Methods in Enzymology* (Lowenstein, J.M., ed.), Vol. 8, pp. 115-117, Academic Press, New York, NY.
9. Berglund, L., Bjorkhem, I., and Einarsson, K. (1982) *Biochem. Biophys. Res. Commun. 105,* 288-295.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-275.
11. Aarsman, A.J., van Deenen, L.L.M., and van den Bosch, H. (1976) *Bioorg. Chem. 5,* 241-253.
12. Agranoff, B.W. (1962) *J. Lipid Res. 3,* 190-196.
13. Hauser, G., and Pappu, A.S. (1982) in *Phospholipids in the Nervous System* (Horrocks, L., ed.), Vol. 1, pp. 283-300, Raven Press, New York, NY.
14. Martin, A., Hopewell, R., Martin-Sanz, P., Morgan, J.E., and Brindley, D.N. (1986) *Biochim. Biophys. Acta 876,* 581-591.
15. Koul, O., and Hauser, G. (1987) *Arch. Biochem. Biophys. 253,* 453-461.
16. Martin, A., Hales, P., and Brindley, D.N. (1987) *Biochem. J. 245,* 247-355.

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COMMUNICATIONS

Increased Amounts of Cholesterol Precursors in Lipoproteins After Ileal Exclusion

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Serum cholesterol precursor sterols reflect the activity of cholesterol synthesis. In this study, squalene, methyl sterol and lathosterol contents were studied in very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) of heterozygous familial hypercholesterolemia patients without and with ileal bypass. The contents of lathosterol and all methyl sterols (lanosterol, $\Delta^{8,24}$-dimethylsterol, Δ^{8}-dimethylsterol, Δ^{8}-methostenol and methostenol), but not of squalene were increased in all lipoproteins by ileal bypass. The increase in the free methyl sterols was more marked than that in the esterified ones. The percentage esterification of the methyl sterols was highest in HDL and lowest in VLDL. Lipoprotein methyl sterol contents were positively correlated with each other and with cholesterol synthesis. The methyl sterols were slightly concentrated in LDL, and squalene strongly concentrated in VLDL. It is concluded that long-term stimulation of cholesterol synthesis increases the methyl sterols in all lipoproteins. *Lipids 23,* **993–996 (1988).**

Serum cholesterol precursor sterols reflect the activity of cholesterol synthesis (1,2), as also evidence by the correlation between the precursors and the hepatic hydroxymethylglutaryl coenzyme A (HMG CoA) reductase activity (3). Therefore, the measurement of these precursors in serum can be used in the detection of clinical states with cholesterol or bile acid malabsorption (4,5) and in the evaluation of changes in different steps of cholesterol synthesis even during short-term processes (6,7).

The bulk of the precursors is transported in low density lipoprotein (LDL). In normal man, the precursor concentrations show a diurnal variation in all three major lipoproteins (6,8). Chronic stimulation of cholesterol synthesis by ileal bypass or resection increases the serum total and LDL precursors (2,5,8–11). It is not exactly known, however, if the changes are similar in the different lipoproteins. Therefore, we studied the effect of the ileal bypass operation (12) on lathosterol, methylated cholesterol precursor sterols and squalene in the different lipoproteins of patients with heterozygous familial hypercholesterolemia.

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Abbreviations: Cholesterol (cholest-5-en-3β-ol); Δ^{8}-dimethylsterol (4,4-dimethyl-5α-cholest-8-en-3β-ol); $\Delta^{8,24}$-dimethylsterol (4,4-dimethyl-5α-cholest-8(7), 24-dien-3β-ol); HDL, high density lipoprotein; HMG-CoA, hydroxymethylglutaryl coenzyme A; lanosterol (4,4,14α-trimethyl-5α-cholest-8,24-dien-3β-ol); lathosterol (5α-cholest-7-en-3β-ol); LDL, low density lipoprotein; methostenol (4α-methyl-5α-cholest-7-en-3β-ol); Δ^{8}-methostenol (4α-methyl-5α-cholest-8-en-3β-ol); VLDL, very low density lipoprotein; GLC, gas liquid chromatography; TLC, thin layer chromatography.

PATIENTS AND METHODS

Patients. Thirteen patients with heterozygous familial hypercholesterolemia, as judged clinically (lipid profiles, xanthomas, family history), were studied as outpatients on their habitual home diet. None of them was taking hypolipidemic drugs. Seven patients (3 men, 4 women) had undergone an ileal bypass operation (12), 6–12 years earlier. Six unoperated control patients (3 men, 3 women) had entered the follow-up of our lipid clinic during the same period of time. Both patient groups were of similar age. Preoperatively, the serum cholesterol concentration and body weight had been similar in both groups, but the control patients were slightly more obese during the present study (data not shown). The purpose and design of the study were explained to the patients and they participated as volunteers. The studies have been approved by the ethics committee of the hospital.

Methods. During the seven-day study period, the patients kept a food record for the calculation of cholesterol intake (13). Similar methods have been shown to give a reasonably valid estimate of dietary cholesterol intake (14). The stools of the final three days were collected for the measurement of fecal bile acids (15) and neutral steroids (16). Cholesterol synthesis was calculated as the difference between cholesterol intake and the sum of fecal neutral steroids and bile acids.

Very low density lipoprotein (VLDL) ($d < 1.006$ g/ml) was isolated by ultracentrifugation (17). High density lipoprotein (HDL) was separated from the infranatant with heparin-manganese precipitation (18). LDL values were calculated as the difference between those of the infranatant and HDL. Separation of squalene, free and esterified cholesterol and methyl sterols by thin layer chromatography (TLC) and the subsequent quantitation with gas liquid chromatography (GLC) have been described earlier (6).

The packed GLC column used in the present study caused difficulties in the detection of lathosterol in patients with normal cholesterol synthesis because, unlike the method sterols, lathosterol could not be concentrated by TLC, separately from cholesterol. In patients with ileal bypass, however, the lathosterol contents of all lipoproteins could be quantitated easily. Therefore, lathosterol values are given only for the operated patients (Table 1). Triglycerides were measured with a fluorometric method (19). The squalene and methyl sterol values are expressed in terms of μmol/mol cholesterol to indicate the lipoprotein precursor content.

Statistical analysis of the data was performed using BMDP Statistical Software (BMDP Statistical Software Inc., Los Angeles, CA).

RESULTS

Ileal bypass had lowered serum total and LDL cholesterol and increased HDL cholesterol (Table 1). Squalene was

TABLE 1

Lipoprotein Cholesterol (mmol/l), Squalene, Lathosterol and Methyl Sterols (μmol/mol Cholesterol)

	Control (n = 6)				Bypass (n = 7)			
	VLDL	LDL	HDL	Total	VLDL	LDL	HDL	Total
Cholesterol	0.37 ± 0.05	8.76 ± 0.55	0.70 ± 0.08	9.83 ± 0.57	0.53 ± 0.07	5.80 ± 0.47	1.02 ± 0.12	7.35 ± 0.43
Squalene	233 ± 83	43 ± 17	52 ± 10	51 ± 17	357 ± 154	34 ± 10	39 ± 8	60 ± 20
Lanosterol	88 ± 17	95 ± 14	87 ± 14	94 ± 14	435 ± 57	371 ± 25	304 ± 28	368 ± 23
$\Delta^{8,24}$-Dimethylsterol	36 ± 10	65 ± 9	34 ± 7	62 ± 9	344 ± 72	329 ± 32	198 ± 22	314 ± 30
Δ^{8}-Dimethylsterol	74 ± 27	102 ± 18	57 ± 16	98 ± 17	513 ± 76	577 ± 70	415 ± 36	552 ± 63
Δ^{8}-Methostenol	88 ± 18	110 ± 15	78 ± 14	107 ± 15	551 ± 78	642 ± 68	558 ± 51	625 ± 65
Methostenol	49 ± 12	99 ± 17	76 ± 15	96 ± 17	168 ± 28	210 ± 28	193 ± 20	205 ± 26
Total methyl sterols	335 ± 57	472 ± 63	333 ± 51	457 ± 62	2012 ± 293	2129 ± 206	1668 ± 102	2065 ± 190
Lathosterol	—	—	—	—	3581 ± 911	2598 ± 612	2251 ± 507	2625 ± 619
Ester (%)					28 ± 4	43 ± 5	56 ± 6	43 ± 5

Mean ± SEM. Squalene and VLDL cholesterol were similar, but other values were changed by ileal bypass ($P < 0.05$ at least, t-test). Lathosterol was not quantitated in the control patients (see the Methods section).

accumulated in VLDL, but the lipoprotein squalene contents were unaffected by the ileal bypass.

The free methyl sterols were up to 10 times increased in all three lipoprotein fractions of the operated subjects. The increases of free Δ^{8}-dimethylsterol and methostenols were higher than those of the esterified ones, resulting in a lowered percentage esterification in the patients with ileal exclusion (Fig. 1). The percentage esterification of the methyl sterols was highest in HDL followed by LDL and VLDL. Compared with the up to 20-fold differences in the sterol concentrations between the different lipoproteins, the contents in terms of μmol/mol cholesterol were remarkably similar in the different lipoproteins. Analysis of variance showed, however, that the methyl sterol contents were slightly higher in LDL than HDL ($P < 0.05$), and tended also to be higher than in VLDL, especially in the control patients. The lathosterol content was markedly increased in the operated patients, and was slightly higher than the sum of the methyl sterols.

Squalene was unassociated with cholesterol synthesis, whereas the methyl sterol contents of all lipoproteins were positively correlated with cholesterol synthesis (Table 2, Fig. 2), especially when the two groups were combined. Methyl sterols of different lipoproteins also were positively associated with each other, and LDL squalene was related to VLDL squalene (Table 3).

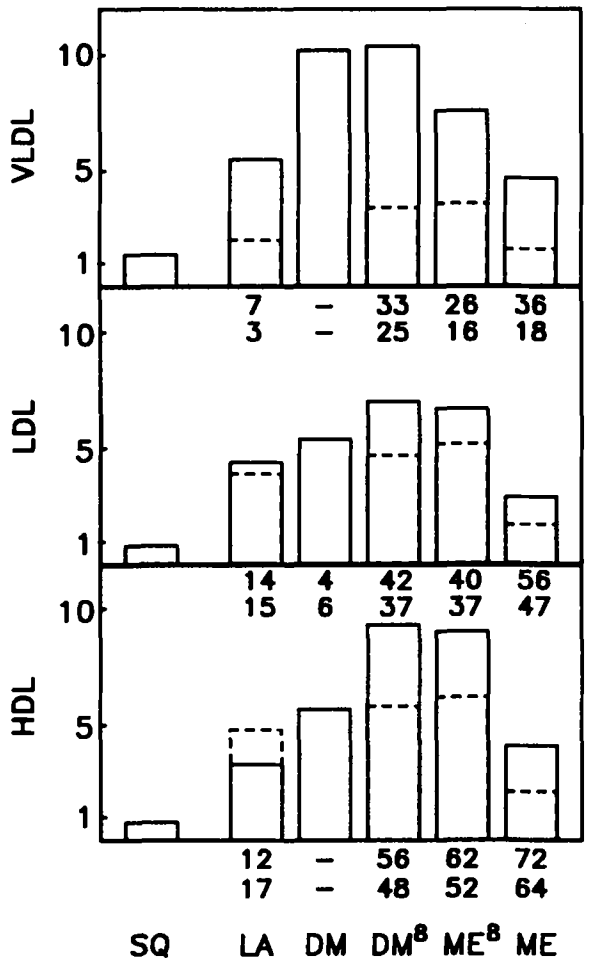

FIG. 1. Relative increases in lipoprotein content of squalene and free (continuous bars) and esterified (broken bars) methyl sterols in patients with ileal exclusion (mean value of the operated patients divided by the mean value of the control patients). Esterified $\Delta^{8,24}$-dimethylsterol was too low for exact detection in controls. Esterification percentage presented below bars for controls (upper rows) and operated (lower rows) patients. SQ = squalene, LA = lanosterol, DM = $\Delta^{8,24}$-dimethylsterol, DM^{8} = Δ^{8}-dimethylsterol, ME^{8} = Δ^{8}-methostenol and ME = methostenol.

DISCUSSION

The present study shows that (a) chronic stimulation of cholesterol synthesis leads to an increase in methyl sterols in all three major lipoprotein classes, (b) that the methyl sterol contents of different lipoproteins are closely correlated and (c) that the distribution of the methyl sterols among the major lipoprotein fractions is quite similar to that of cholesterol, although these sterols are somewhat concentrated in LDL. In addition, the lack of association between the squalene content of any lipoprotein and cholesterol synthesis confirms earlier reports that the serum squalene content is not a reliable index of cholesterol synthesis activity (2,11,20,21).

Earlier studies on the effect of stimulation of cholesterol synthesis by different methods have revealed an

TABLE 2

Correlation Between Cholesterol Synthesis (μmol/kg/day) and Lipoprotein Squalene and Methyl Sterol Content (μmol/mol Cholesterol)

	All (n = 13)	Control (n = 6)	Bypass (n = 7)
Squalene	.160	.380	.114
VLDL	.175	.148	−.051
LDL	−.076	.549	−.032
HDL	−.405	−.386	−.445
Lanosterol	.949[a]	.190	.673
VLDL	.827[a]	.579	.156
LDL	.951[a]	.135	.704
HDL	.868[a]	.470	.227
Sum methyl sterols	.902[a]	.508	.351
VLDL	.783[a]	.989[a]	−.038
LDL	.906[a]	.469	.408
HDL	.921[a]	.662	.267

[a]P < 0.001.

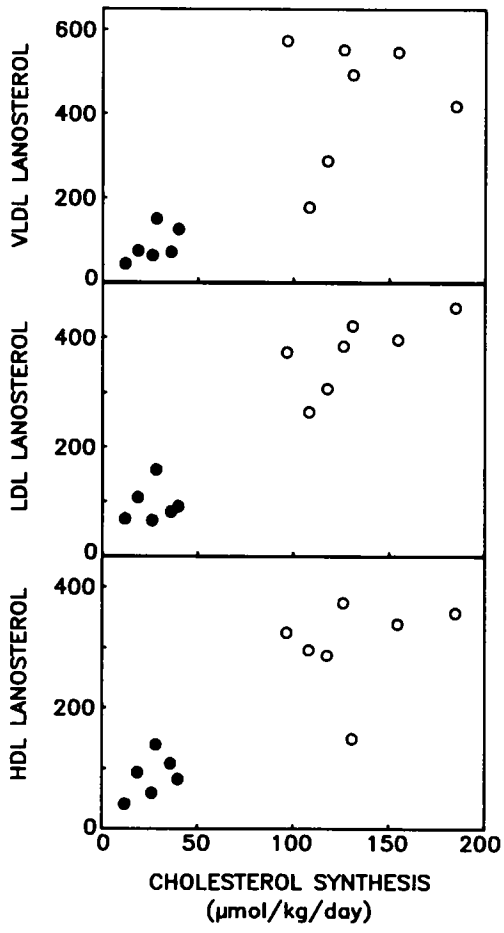

FIG. 2. Correlation of cholesterol synthesis (μmol/kg/day) with lipoprotein lanosterol content (μmol/mol cholesterol). ●, Control patient; ○, patient with ileal bypass.

TABLE 3

Correlation of LDL Squalene and Methyl Sterol Content (μmol/mol Cholesterol) with Squalene and Respective Methyl Sterol Content (μmol/mol Cholesterol) in VLDL and HDL

	All (n = 13)	Control (n = 6)	Bypass (n = 7)
LDL squalene			
VLDL squalene	.674[b]	.646	.983[c]
HDL squalene	.107	−.289	.680
LDL lanosterol			
VLDL lanosterol	.929[c]	.817[a]	.721
HDL lanosterol	.862[c]	.861[a]	.017
LDL $\Delta^{8,24}$-dimethylsterol			
VLDL $\Delta^{8,24}$-dimethylsterol	.908[c]	.367	.813[a]
HDL $\Delta^{8,24}$-dimethylsterol	.780[c]	.362	−.201
LDL Δ^{8}-dimethylsterol			
VLDL Δ^{8}-dimethylsterol	.873[c]	.151	.548
HDL Δ^{8}-dimethylsterol	.965[c]	.573	.874[b]
LDL Δ^{8}-methostenol			
VLDL Δ^{8}-methostenol	.923[c]	.198	.705
HDL Δ^{8}-methostenol	.992[c]	.925[c]	.968[c]
LDL methostenol			
VLDL methostenol	.853[c]	.427	.766[a]
HDL methostenol	.978[c]	.976[c]	.984[c]

[a]P < 0.05.
[b]P < 0.01.
[c]P < 0.001.

increment in VLDL and LDL methyl sterols, less so in HDL (8,11). The present study shows that the methyl sterol content of HDL also is increased in these patients. The finding that the diurnal variation in the methyl sterol content of LDL is similar to that of HDL (6) fits to the present ones, namely that the methyl sterol content of LDL is more closely correlated with that of HDL than that of VLDL.

The turnover of the methyl sterols and the diurnal changes in lipoprotein methyl sterol content are faster than the turnover of the lipoprotein particles (2,6,11). Therefore, it has been proposed that the methyl sterols might be released to the different lipoprotein particles directly from the hepatocyte cellular membrane, and may be taken up by the liver from the bypassing lipoprotein particles (11). This hypothesis could explain the similarities among the methyl sterol contents of the different lipoproteins seen in the present study. The finding on methyl sterol release on and off lipoproteins is interesting evidence that lipoprotein sterols, probably including cholesterol, can be metabolized without the catabolism of whole lipoprotein particles.

The percentage esterification of the methyl sterols was highest in HDL and lowest in VLDL. The reason for this finding is not obvious. Unlike cholesterol, the methyl sterols are not esterified in serum (22). Therefore, the methyl sterol esters seem, for some reason, to be preferentially concentrated in the heavier lipoprotein particles.

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REFERENCES

1. Miettinen, T.A. (1969) *Life Sci. 8,* 713-721.
2. Miettinen, T.A. (1970) *Ann. Clin. Res. 2,* 300-320.
3. Björkhem, J., Miettinen, T., Reihner, E., Ewerth, S., Angelin, B., and Einarsson, K. (1987) *J. Lipid Res. 28,* 1137-1143.
4. Vuoristo, M., and Miettinen, T.A. (1986) *Gut 27,* 1312-1319.
5. Färkkilä, M.A., Tilvis, R.S., and Miettinen, T.A. (1988) *Gut 29,* 188-195.
6. Miettinen, T.A. (1982) *J. Lipid Res. 23,* 466-473.
7. Miettinen, T.A. (1988) *J. Lipid Res. 29,* 43-51.
8. Miettinen, T.A. (1971) *Ann. Clin. Res. 3,* 264-271.
9. Miettinen, T.A. (1971) *Eur. J. Clin. Invest. 1,* 452-460.
10. Miettinen, T.A., and Koivisto, P. (1983) in *Bile Acids and Cholesterol in Health and Disease* (Paumgartner, G., Stiehl, A., and Gerok, W., eds.) pp. 183-187, MTP Press, Lancaster, England.
11. Miettinen, T.A. (1985) *Metabolism 34,* 425-430.
12. Buchwald, H., and Varco, R.L. (1966) *J. Am. Med. Assoc. 196,* 627-630.
13. Ahlström, A., Räsänen, L., and Kuvaja, K. (1972) *Ann. Acad. Sci. Fenn., A IV Biol. 194,* 1-8.
14. White, E.C., McNamara, D.J., and Ahrens, E.H. Jr. (1981) *Am. J. Clin. Nutr. 34,* 199-203.
15. Grundy, S.M., Ahrens, E.H. Jr., and Miettinen, T.A. (1965) *J. Lipid Res. 6,* 397-410.
16. Miettinen, T.A., Ahrens, E.H. Jr., and Grundy, S.M. (1965) *J. Lipid Res. 6,* 411-424.
17. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) *J. Clin. Invest. 34,* 1345-1353.
18. Lipid Research Clinics Program (1974) *Manual of Laboratory Operations,* Vol. 1, pp. 51-59, DHEW Publication No. (NIH) 75-628, Bethesda, MD.
19. Kessler, G., and Lederer, H. (1966) in *Automation in Analytical Chemistry* (Skeggs, L.T., ed.) pp. 341-344, Medical Inc., New York, NY.
20. Liu, G.C.K., Ahrens, E.H. Jr., Schreibman, P.H., and Crouse, J.R. (1976) *J. Lipid Res. 17,* 38-45.
21. Saudek, C.D., Frier, B.M., and Liu, G.C.K. (1978) *J. Lipid Res. 19,* 827-835.
22. Tilvis, R.S., and Miettinen, T.A. (1980) *Scand. J. Clin. Lab. Invest. 40,* 671-674.

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Lipids

Notices
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Genetic Basis for Obesity and Diabetes (October 30–November 1, 1988, Stouffer Pineisle Resort, Lake Lanier Islands, Georgia). Sponsored by the University of Georgia, Center for Continuing Education and the College of Home Economics. For more information, contact Dr. Carolyn D. Berdanier, Conference Chair, University of Georgia, Department of Foods and Nutrition, Dawson Hall, Athens, GA 30602.

Vitamin E: Biochemistry and Health Implications (October 31–November 3, 1988, The Sheraton Centre, New York, NY). Sponsored by The New York Academy of Sciences. For more information, contact the Conference Department, The New York Academy of Sciences, 2 East 63rd St., New York, NY 10021, (212) 838-0230.

International Symposium on Clinical, Biochemical and Molecular Aspects of Fatty Acid Oxidation (November 6–9, 1988, Penn Tower Hotel, Philadelphia, PA). For more information, contact Paul M. Coates, Ph.D., Division of Genetics, The Children's Hospital of Philadelphia, 34th St. and Civic Center Blvd., Philadelphia, PA 19104.

23rd Annual Hugh Lofland Conference on Arterial Wall Metabolism (May 24–27, 1989, Welches, Oregon). Requests for information should be sent to M. R. Malinow, M.D., Chairman, Hugh Lofland Conference, Oregon Regional Primate Research Center, 505 NW 185th Avenue, Beaverton, OR 97006, (503) 690-5258.

10th Anniversary Meeting of the European Association for Cancer Research (University College, Galway, Ireland, September 11–13, 1989). For further information, contact Dr. S. M. Lavelle, Experimental Medicine, University College, Galway, Ireland; or in North America, contact Dr. J. H. Weisburger, American Health Foundation, Valhalla, New York, NY 10595-1599. The program involves plenary lectures, workshops, symposia and poster sessions.

REVIEW

Absorption and Transport of Fat in Mammals With Emphasis on n-3 Polyunsaturated Fatty Acids

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The current state of knowledge concerning the absorption and transport of dietary fat with emphasis on long-chain polyunsaturated n-3 fatty acids in mammals is reviewed. It is apparent that long-chain polyunsaturated n-3 fatty acids, either as free acids or as part of triglycerides, are readily absorbed in the gut and transported by the circulatory system. Indeed, it would appear that long-chain polyunsaturated n-3 fatty acids are digested, absorbed and transported similarly to other long-chain fatty acids with only minor variations, although there is much that is still not understood about these processes. The main unresolved issues in the area of the absorption and transport of long-chain polyunsaturated n-3 fatty acids appear to be: 1) If they, when located in the 2-position of triglycerides, have unique metabolic pathways; and 2) whether the unnatural forms, i.e., methyl or ethyl ester derivatives, are suitable vehicles for administration as dietary supplements. The effect in man of dietary, long-chain polyunsaturated n-3 fatty acids on blood serum lipid and lipoprotein levels, particularly the low density lipoproteins, remains controversial, except for the well-documented reduction in serum triglyceride levels. Also, there is uncertainty regarding their distribution and metabolism in tissues. Finally, if the consumption of long-chain polyunsaturated n-3 fatty acids has beneficial health consequences, what is the appropriate therapeutic dose? In view of these important, unresolved issues and uncertainties, it would seem prudent to direct additional research toward a better understanding of the overall process by which fat is digested, absorbed and transported.
Lipids 23, **1005–1014 (1988).**

Within the last two decades, an intense interest has developed in the biochemistry, physiology and metabolism of the family of polyunsaturated fatty acid derived from alpha-linolenic acid, the so-called omega-3, or n-3 or, simply, n3 family (1–4). This interest was stimulated largely by the report of Bang and Dyerberg (5), stating that Eskimos in Greenland had a much lower rate of heart disease than people living in Denmark, despite the very high fat consumption by the Eskimos. Dyerberg (6,7) suggested that the n-3 fatty acids, particularly eicosapentaenoic acid (EPA), may be the primary protective agent in the Eskimo diet. Although this assumption has not been proven valid in some animal models to the satisfaction of all investigators (8,9), it has been established that EPA and other n-3 fatty acids do prolong bleeding times in humans (10–13) and some other animal species (9,14), reduce the tendency of platelets to aggregate (14–22), as well as influence blood viscosity (23–25) and platelet fluidity (26). Monkeys, when fed fish oil diets, show a marked decrease in cardiac arrhythmias (27). Other investigators have reported protective effects of dietary n-3 fatty acids in experimental atherosclerosis (21,28–30). Kromhout et al. reported that individuals who consumed fish in modest amounts had less heart disease than nonfish eaters in a free-living population (31). Storlien et al. reported in 1987 that a diet containing fish oil prevented the development of insulin resistance in rats consuming a high-fat diet (32).

The human dietary requirement for n-3 fatty acids, if indeed they are an essential dietary nutrient (1,2,33–37), is not known at present, although a recent study by Bjerve et al. (38) suggested that the human requirement of alpha-linolenic acid is ca. 0.2% of the total energy intake per day.

Fatty acids of the n-3 series are part of natural foods and are usually eaten in the form of triglyceride (39). The assumption of most investigators has been that n-3 fatty acids are digested, absorbed and transported through normal digestive processes (40,41) and similarly to other types of long-chain fatty acids in the diet. However, although there has been little or no specific experimentation to test this hypothesis, a body of experimental work is developing that allows a critical look at this assumption (42–47).

This area of research includes the use of ethyl esters of menhaden-oil fatty acids, or of a concentrate (ca. 80% total n-3 fatty acids from menhaden-oil fatty acids, also in ethyl ester form). These are provided gratis to NIH or similar peer-review projects by the National Marine Fisheries Service of the Department of Comerce (J. D. Joseph, private communication). At the popular or consumer level, the enriched natural triglyceride oils include 300 mg/g of EPA + docosahexaenoic acid (DHA), as well as higher concentrations in the form of ethyl and methyl esters and/or free acids (48). It can be assumed that more concentrated oils will probably be triglycerides produced from enriched acids or esters, presumably by direct enzyme transesterification. These will be abnormal triglycerides in the context of the structures of either fish oils and lipids, or of the fatty tissues of marine mammal ingested by the Eskimos (46).

To our knowledge, the subject of absorption and transport of n-3 polyunsaturated fatty acids has not been reviewed previously. Indeed, the paucity of investigations of the specific absorption and transport of all long-chain fatty acids is a serious deficiency in modern nutritional science. Thus, it is timely to briefly review this area for

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Abbreviations: DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FABP, fatty acid binding protein(s); HDL, high density lipoprotein; IDL, intermediate density lipoprotein; PUFA, polyunsaturated fatty acid(s); (V)LDL, (very) low density lipoprotein.

the benefit of the many investigators engaged in feeding fatty acids, particularly n-3 fatty acids, to humans and/or animals.

This review will be limited to a discussion of the digestion and absorption processes of long-chain fatty acids and their transport in the circulatory system of mammals. Metabolic interconversion and utilization or health effects will not be covered, as these areas have been reviewed extensively elsewhere (1–3,36,39,49–53). Indeed, as this review cannot be totally comprehensive, it is intended to be more of an overview, highlighting specific topics and calling attention to areas where opinions are divided or significant gaps in knowledge currently exist.

OUTLINE OF BIOCHEMISTRY AND PHYSIOLOGY OF FAT ABSORPTION AND TRANSPORT

Fat absorption is a multistage metabolic process, the complexity of which is compounded by the insolubility of most lipids in aqueous medium (54). When fat is consumed, either in natural foods or in a purified form (in experimental dietary protocols), a series of physiological events are activated that last for 16 to 24 hr, if no other food is consumed after the initial meal (55). The majority of the fatty acids in the human diet, whether of vegetable or animal origin, is of the C_{18}-chain length, with lesser quantities of C_{16}-chain length, almost invariably palmitic acid (16:0). Butter fat with 10 mol % butyric acid is an exception.

Coconut oil, with 70–80% (w/w) fatty acids of C_8–C_{14}-chain length, is an important dietary element to some population groups. Mustardseed oil and the older, high-erucic acid rapeseed oils are also still important in some regions and have 10–20% of 20:1 and 20–50% of 22:1. Fish oils span the chain-length range from C14 to C22. Unfortunately, some researchers have used the term "medium-chain length" for the C_8–C_{14} group, so that, by implication, the common C_{16}–C_{18} fatty acids became long-chain length. In keeping with the need to distinguish the longer-chain polyunsaturated fatty acids (PUFA), such as arachidonic (20:4n-6), EPA (20:5n-3) and docosapentaenoic (DPA) (22:5n-3) acids, from the C_{18} PUFA, such as 18:2n-6 or 18:3n-3, our terminology will assume that C_4–C_{12} = short chain, C_{14}–C_{18} = medium chain and C_{20}–C_{24} = long chain.

Initially, the fatty food, if consumed orally (rather than as capsules or by stomach tube), is mixed with lingual lipase and hydrolysis of the triglycerides begins in the stomach (56,57). There is also a gastric lipase (57,58) that, particularly in humans, may be more important than lingual lipase in the initial hydrolysis of fat. DeNigris (58) has reported that lingual lipase is not a major source of fat hydrolysis in humans, but is important in other species. Conversely, Moreau et al. (57) indicated in a recent report that gastric lipase is the major enzyme in human gastric juice. It is possible that the gastric lipase is more specific for short- and medium-chain triglycerides, or those with mixed chain lengths (59).

Very short-chain (C < 8 or 10) fatty acids are absorbed directly from the stomach into the venous circulation (60–62); C_{14}- to C_{18}-chain-length fatty acids, as free or esterified fatty acids, are passed mainly into the intestinal lumen where pancreatic lipase, colipase and bile are added to the digestive ferment, and further lipolysis takes place. It is not clear if any significant portion of the free fatty acids liberated, e.g., those with chain lengths greater than 14 carbons, are absorbed into the venous circulation from the stomach. The studies of Chen and coworkers (42–45) would suggest, however, that the majority of the n-3 fatty acids are passed into the small intestine from the stomach, where absorption takes place.

Lingual lipase and pancreatic lipase liberate fatty acids from the 1 and 3 positions of natural triglycerides, leaving the 2-monoglyceride intact (63,64). The positional specificity of gastric lipase has not been elucidated yet (57). Phospholipids are also hydrolyzed by pancreatic enzymes to free fatty acid and their derived lysocompounds (64,65). Pancreatic phospholipase A_2 acts on the 2 position of phospholipids to produce 1-lysocompounds. Pancreatic lipase (1,3-acylglycerol ester hydrolase) will act on the 1 position of phospholipids also (63,64); however, it is generally assumed that most phospholipids are absorbed by the enterocyte as the 1-lysocompound after being hydrolyzed by phospholipid A_2 (64,66). Cholesteryl esters, if present, are hydrolyzed completely (66) to free cholesterol and free fatty acids. Sphingomyelin, usually a minor component of the diet, can be absorbed intact (66).

It should also be noted that some long-chain free fatty acids are absorbed by the portal circulation (60,61,67,68), but the amount absorbed by this route may vary with types and amounts of other nutrients being digested concurrently (69). There is, as yet, no evidence to suggest that significant quantities of the n-3 polyunsaturated fatty acids enter the circulation through the portal vein system (43,44,70), but experiments have not yet included entry by this route for fatty acids from ethyl or methyl esters.

Once the normally ingested fats have been hydrolyzed to free fatty acids, monoglycerides, lysophospholipids and free cholesterol, the hydrolysis products are absorbed by the enterocytes of the intestinal wall. The process of absorption from the intestinal lumen into the enterocyte is by passive diffusion (69). Free fatty acids, lysophospholipids and 2-monoglycerides can be absorbed almost completely in the absence of bile salts (70), although the presence of bile is necessary for the absorption of free cholesterol (71).

In the enterocyte, the lipolysis products are reesterified (72). Cholesterol can be synthesized de novo, if there are inadequate dietary quantities, but even if no cholesterol is supplied by the diet, the bile supplies appreciable quantities to the enterocyte during fat absorption (70,71). The newly synthesized triglycerides, phospholipids and cholesteryl esters are combined with de novo synthesized apolipoproteins, mainly apo B-48, with some apo AI, AII, and AIV, and apo E, to form nascent chylomicrons (73). The chylomicrons are then secreted into the lymphatic system and carried by the thoracic lymphatic duct to the vena cava where they enter the circulation (73). The chylomicrons interact with the serum lipoproteins, absorbing additional apoproteins and albumin and exchanging phospholipids and free fatty acid (74–76). This process produces the mature chylomicron that interacts with circulating lipoprotein lipase which is released from the capillary endothelial cells (77). Lipoprotein lipase initially hydrolyzes chylomicron triglycerides to free fatty acids and 2-monoglycerides.

The liver does not clear chylomicrons directly (78). Rather, their core lipids are hydrolyzed first by the

lipoprotein lipase-colipase system to form chylomicron remnants that are then cleared from the circulation by the apo E receptor (79). Apo B-48 does not bind to the low density lipoprotein (LDL) receptor (80). The chylomicron remnant consists of apo B-48, apo E, phospholipids, cholesterol, cholesteryl ester and some triglyceride (81).

Lipoprotein lipase is activated by apo C-II (77). It has positional specificity for the primary ester bonds of acylglycerols (82) and hydrolyzes the triglycerides to 1,2[2,3]-diacylglycerols and free fatty acids. It also hydrolyzes 1,2[2,3]diacylglycerol to 2-monoacylglycerol and 1,[3]-monoglycerides to free glycerol and fatty acids (83). Lipoprotein lipase has no effect on 2-monoglycerides (78); 2-monoglycerides are hydrolyzed by lipases in the liver (74). This observation may have important implications for absorption, transport and metabolism of n-3 fatty acids as the long-chain n-3 fatty acids may be preferentially distributed in certain positions of the triglyceride molecules (48,84,85). Lipoprotein lipase also hydrolyzes the primary acylester bond of phosphatidylcholine to the 2-acyl lyso-compound and a free fatty acid (86).

The lipoprotein lipase hydrolysis takes place in the peripheral tissues in contact between the chylomicron and the endothelial cells, and the free fatty acids released are taken up by the proximal tissue (76). Some of the free fatty acids released by lipoprotein lipase undoubtedly become bound to albumin and are cleared by the liver (87). As the size of the chylomicron is reduced through hydrolysis of its triglycerides, the apoprotein-phospholipid-cholesteryl coat forms "buds" which apparently split off to form nascent high density lipoprotein (HDL) particles (88). There is no triglyceride or cholesterol ester in these particles that consists exclusively of apo E, apo AI, apo AII and apo C, along with phospholipid and unesterified cholesterol. These nascent HDL particles then acquire cholesteryl esters through the action of the lecithin-cholesterol acyl transferase in blood (89). HDL particles, probably HDL_2, are cleared by the liver (89,90), although there is still controversy over this hypothesis (74,89,90).

In the eucaloric or excess caloric condition, the liver catabolizes chylomicron remnants, synthesizes triglycerides from carbohydrate, and forms very low density lipoproteins (VLDL) that are released into the circulation (74). VLDL is also a substrate for endothelial lipoprotein lipase. VLDL loses triglycerides by hydrolysis and is transformed to intermediate density lipoproteins (IDL) and LDL. LDL is taken up by the LDL receptor (91) of peripheral tissue and the liver. LDL primarily transports cholesteryl esters to the peripheral tissues, where they are hydrolyzed to free cholesterol and then reacylated (92).

ABSORPTION OF N-3 FATTY ACID

The foregoing was a brief overview of normal fat absorption and transfer in mammals, with the exception of ruminants, that absorb fat somewhat differently (93,94). However, in the above discussion, the dietary fatty acids were treated as if all were physiologically equal. This is obviously not true, yet it is an implicit assumption made in most discussions of the subject. First, digestive lipases may not hydrolyze triglycerides containing n-3 fatty acids as efficiently as triglycerides not containing this type of fatty acid (84,85,95–97). Second, there may be differential absorption of n-3 long-chain polyunsaturated fatty acids liberated by the digestive lipases, compared with either saturated, monounsaturated or n-6 polyunsaturated fatty acids. This will be discussed later.

More than twenty years ago Brockerhoff (84,95), Yurkowski and Brockerhoff (96) and Bottino et al. (85) demonstrated that triglycerides containing EPA, DPA (n-6 isomer) and DHA in the 1 and 3 position were resistant to lipolysis by pancreatic lipase in vitro. Brockerhoff (97) later found that the inhibition was specifically produced by the introduction of a double bond in the delta-2 through delta-5 position of the fatty acid chain. Introduction of additional double bonds towards the methylene end of the chain did not increase the inhibition, contrary to the hypothesis of Bottino et al. (85). However, all these investigators reported results obtained primarily with short-term incubations (usually 15 minutes or less) and partial hydrolysis of triglycerides using purified porcine pancreatic lipase and nonphysiological conditions. Although the conditions used in the in vitro experiments bore little resemblance to the in vivo lipolysis of dietary fat, Chen et al. (43) reported that the recovery of fatty acids in the lymph of rats with thoracic cannulas was less complete (57% and 47%, respectively), when fish oil and fish oil concentrates were given, instead of corn oil. Although there have been no direct studies of the efficiency of lipolysis of triglycerides containing n-3 fatty acids in humans, Harris and Connor (98) reported the reduction of postprandial of hypertriglyceridemia in subjects fed a fatty meal, where the source of fat was salmon oil, a result compatible with a slower rate of lipolysis of the n-3–containing triglycerides in the intestine. However, the work of Chen et al. (43–45), El Boustani et al. (47) and Lawson and Hughes (99) suggests that once triglycerides containing n-3 fatty acids are hydrolyzed, the n-3 fatty acids liberated are totally absorbed in vivo. Once absorbed, the n-3 fatty acids appear to be metabolized similarly to other fatty acids. Chen et al. (44,45) found no apparent resistance to hydrolysis of chylomicron triglycerides containing n-3 fatty acids by lipoprotein lipase in rats. Thus, as the digestion of fat may go on for hours in vivo (63,64, 69–71), the rate of lipolysis may have little influence on the ultimate amount of fat absorbed, but the rate of lipolysis could effect the route, i.e., the lymphatic or venous systems, by which the liberated fatty acids enter the circulation (see the discussion of n-3 fatty acid absorption). Although triglycerides containing fatty acids (with double bonds within 5 carbons of the carboxyl end of the molecule, i.e., most n-3 fatty acids) resist pancreatic lipase digestion, further investigation of the action of lipases in vivo on these compounds may be warranted, particularly because it is not known if triglycerides containing n-3 fatty acids are resistant to hydrolysis by lingual or gastric lipases.

It is known that the route of absorption of fatty acids is affected by the length of the carbon chain. Short-chain fatty acids of fewer than 8–12 carbons in chain length are absorbed and pass into the portal vein (61,62). Thus, they go directly to the liver without ever being incorporated into chylomicrons. About one-third to one-half of the fatty acids with carbon-chain lengths of 12 or longer must normally go through the lymphatic-chylomicron route, because only half of the 1- and 3-position fatty acids come

from endogeneous sources (99). The n-3 polyunsaturated fatty acids can be assumed to use this latter route of absorption (100), as they are mainly 18 carbons or longer in chain length (39). The portion absorbed through the portal system (60,61,67,68) seems to profoundly affect liver function and probably explains the well-documented reduction in serum triglycerides in humans (2,98).

The degree of molecular unsaturation has a profound effect on absorption, at least regarding the saturated n-9 and n-6 series of long-chain fatty acids (101–104). In the rat, the saturated fatty acids are poorly absorbed compared with the unsaturated analogs of equivalent chain length (101). Early work by Carroll (101), confirmed by others (61, 102–106), indicated that palmitic acid was absorbed better than stearic acid, and oleic acid was absorbed more completely than either of the saturated acids. Little data are available on this topic in man. Although the poor absorption of the longer-chain saturated fatty acids is presumed due to the higher melting point and great insolubility in an aqueous medium, this has not been explored extensively (54,69). The absorption of fatty acid in the gut does not appear to depend on the bile, because animals with ligated bile ducts appear to absorb fatty acids efficiently (70).

Feeding intact triglycerides in rodents indicate that stearic acid is better absorbed when it is fed as a mixed triglyceride than when fed as tristearin (107,108). Tomarelli et al. reported that an increased stearic acid content of fat decreased the amount of total fat absorbed (109). These authors (109) also found that the amount of palmitic acid in the 1 and 3 positions of the triglyceride reduced total fat absorption. Human infants have been reported to absorb 88% of the stearic acid when fed lard, but only 40% of the stearic acid from lard that had been randomized (110). Recently, Bonanome and Grundy (111) reported essentially complete absorption of stearic acid (>98%) in men fed synthetic triglycerides in a formula diet containing approximately 40% stearic acid, and that the absorption of stearic acid was equivalent to that observed for palmitic and oleic acids incorporated into synthetic triglycerides.

Reports by Chen et al. (42,45) indicate that EPA is absorbed as efficiently as oleic acid in rats and that the absorption of both oleic acid and EPA was equivalent to arachidonic acid. Carroll (101) compared the absorption of saturated and more unsaturated fatty acids and found that longer-chain monounsaturated fatty acids are less well absorbed than those with 18 carbons or less. The work of Chen et al. (42,45) and others (101,105,106) then suggests that additional double bonds in the molecule increase absorption, but that lengthening the chain will decrease the percentage absorbed, compared with a shorter chain having the same number of double bonds.

There is relatively little known about the effect of nutrients such as carbohydrates and proteins on fatty acid absorption. The recent review of physicochemical mechanisms of fat absorption by Carey et al. (69) implied that the presence of other nutrients should enhance absorption compared with a sample of pure fat given as a bolus or by gastric tube. Vallot et al. (61) found that the intestinal pathway of decanoic acid absorption was effected by the presence of other lipids. Vahouny (112) indicated that dietary fiber reduced the absorption of dietary fat. Thus, experiments in which pure fat samples have been used to test absorption may not reflect the situation when natural foods are eaten.

In experimental feeding studies, natural (or synthetic) fats (triglycerides) (102,107–111) are often used as the source of dietary fat, but equally common is the use of purified fatty acid esters, such as the ethyl or methyl ester (7,47,113,114). Although the methyl ester was used in older work (104), it has been replaced, in most instances, by the ethyl ester (42,47,114–120). Occasionally the free fatty acid (120,121) or the arginine salt (47,122) has been used as a delivery vehicle for dietary supplement of the n-3 fatty acids. However, there has been no assurance that the ethyl ester is hydrolyzed by pancreatic lipase as efficiently as natural fats. Indeed, some evidence suggests that ethyl esters are hydrolyzed to a lesser degree than triglycerides or methyl esters (47,99), however, recent work in Japan (114,123), Canada (124), the USA (42) and France (47) shows that ethyl esters of n-3 fatty acids certainly are absorbed by man and rats. Ethyl and methyl esters are relatively rare in natural foods and are most often associated with flavor components (125), although they can be found in small amounts in processed food and alcoholic beverages (126). Thus, assuming absorption and transport identical to that of natural fats may not be correct. There are reports also indicating that fatty acid ethyl esters may be toxic to mitochondria (127,128). The report by El Boustani et al. (47) suggested that ethyl esters of n-3 fatty acids are differentially absorbed in humans, when compared with 2-acyl glycerol EPA, free EPA or its arginine salt. This confirms an earlier report by Nagakawa et al. (212) citing differences in the absorptive biochemistry of free acids or ethyl esters of EPA.

The existing literature on absorption of fats containing n-3 fatty acids suggests they can be readily absorbed as free acid or from triglycerides after hydrolysis by pancreatic, gastric or sublingual lipase (42–45,47,56). Vahouny et al. (129) showed that only half of dietary free EPA appears in the lymph lipids, as similarly reported by El Boustani et al. (47) for ethyl esters. Possibly for ethyl esters, the other half passes through the portal system as a result of in situ hydrolysis after absorption by the brush border in the gut wall. However, EPA as the ethyl ester, although probably absorbed (47) by two different routes, is absorbed totally (123). Selecting which form of EPA is the optimal vehicle (46) for specific n-3-fatty acid supplementation remains equivocal. Although the 2-monoglyceride is absorbed from the lumen and reesterified to triglyceride by the enterocyte without deacylation, it is reported that the ethyl ester does not reach the lymph or circulation intact (121). This suggests that the enterocyte has an esterase or transesterase that can hydrolyze the ethyl ester, allowing incorporation of the liberated fatty acid into de novo synthesized triglycerides in the enterocyte. El Boustani and coworkers's data suggest that this could be the case (47). If pancreatic lipase action on ethyl esters is delayed, then some ethyl ester could be hydrolyzed by intestinal microflora farther down the tract.

As Ackman recently pointed out (46), when n-3 fatty acids are ingested, their molecular structure could have a profound effect on lipid production and its rate, if not the total quantity absorbed. Hence, the physiological and pharmacological properties of n-3 fatty acids could be influenced by the molecular form in which they are ingested.

Although a lot has been written about the effect of dietary n-3 fatty acids on the fatty acid composition of human and other mammalian body fluids and organs (130–136), as well as on eicosanoid metabolism (20,27,39, 115,131,137,138), this review only refers to the data related to absorption and transport of n-3 fatty acids.

The transport of fatty acids from the intestinal lumen is by passive diffusion (63) and takes place in the absence of bile salts. It is likely, but not certain if the 2-monoglyceride is also transported by passive diffusion. Once the free fatty acid has entered the enterocyte, it is rapidly converted by an energy-dependent process to triglycerides and secreted, by another active, energy-dependent process, in the form of nascent chylomicrons. The formation and synthesis of triglycerides takes place in the endoplasmic reticulum (139,140). The free fatty acids are activated to their CoA derivative and the "triglyceride synthetase" complex then incorporates the fatty acid moiety of acyl-CoA unit into triglycerides (139,141). Alternatively, the d-glycerophosphate pathway can also be active in the synthesis of mucosal triglycerides and phospholipids (139,142). The nascent chylomicron particles are formed in the Golgi apparatus (52,139,143). There is reason to believe that apo B-48 is being continuously synthesized in enterocytes, but that its synthesis is stimulated further by dietary fat intake (144). Apo B is not synthesized in the Golgi apparatus but in the rough endoplasmic reticulum (144) and transported to the Golgi apparatus by an undefined mechanism. Thus, only assembly and secretion of the chylomicrons takes place in the Golgi apparatus. However, some glycosylation of the nascent chylomicrons takes place in the Golgi apparatus (145). Actual secretion of the chylomicrons into the lymph takes place by exocytosis from chylomicron-filled vesicles that "bud-off" from the Golgi-cisternae.

Dietary n-3 fatty acids lower serum triglyceride levels (12,19,146–150) apparently by reducing liver triglyceride production and VLDL secretion (151–154). It is not known if n-3 fatty acids also inhibit triglyceride synthesis in the enterocyte, but it is known that fat meals high in n-3 fatty acids do not usually produce the hyperlipemia characteristic of meals high in saturated fat (2,98). Marine mammals do, however, exhibit chylomicronemia when consuming food rich in n-3 fatty acids (155).

Thus, a simplified scheme for the absorption, processing and excretion of fat in the enterocyte is as follows: 1) Fatty acids, 2-monoglycerides, lysolecithin and free cholesterol are passively absorbed into the enterocyte by the microvilli of the brush border; 2) triglycerides, cholesteryl esters and lecithin along with apolipoproteins (apo B-48, apo AI and apo AII, apo AIV and apo C) are synthesized in the endoplasmic reticulum; 3) the lipids and apoproteins are combined into prechylomicron particles either before or in the Golgi apparatus; and 4) nascent chylomicrons are secreted into the lymph by exocytosis.

TRANSPORT OF N-3 FATTY ACIDS

The above description ignores the role of specific fatty acids in this process. Indeed, the tacit assumption has been made that, because a wide variety of fatty acids are encountered in the natural state and are necessary for normal metabolism, the absorption process ideally should be independent of the particular variety of fatty acid being consumed. This would be evolutionarily conservative and prudent. As noted above, there is evidence to the contrary (61,101–106). Long-chain saturated fatty acids are poorly absorbed, perhaps due to calcium soap formation and, at high concentrations, linolenic acid may also be poorly absorbed (Nelson, unpublished observations). At lower intake levels, however, alpha-linolenic acid is readily absorbed and may cause a significant enrichment in the n-3 fatty acid content of tissue lipids, as reported recently by O'Dea et al. (156). It is not clear why the longer-chain saturated fatty acids are not absorbed except that, being very hydrophobic, they would not be expected to readily form micelles. Also, they may not penetrate the membrane of the enterocyte, again due to their hydrophobic characteristics (51,157). However, there is good reason to believe that the absorption phenomena is dose-dependent and, under normal physiological circumstances, during the consumption of natural foods, long-chain saturated fatty acids are not discriminated against during the absorption process (69,70,111).

It is also possible that n-3 fatty acids may be asymmetrically distributed in triglycerides and phospholipids of chylomicrons formed during fat absorption (158–160). They appear to be asymmetrically distributed in tissue phospholipids and, perhaps, triglycerides. It is equally possible that this asymmetry is extended to the distribution and metabolism in the liver (161) and other target organs (124,162,163), as well as in the circulating lipoproteins (164,165).

However, Chen et al. (44) have shown that eicosapentaenoic acid and chylomicrons formed when this n-3 fatty acid is given to rats are cleared from the circulation with the same time curve as oleic acid. The same group has reported (43) similar findings when menhaden oil, fish oil concentrates and corn oil are fed to rats. This would indicate that the extrahepatic lipoprotein lipase does not discriminate against n-3 fatty acids, nor is the uptake of the free fatty acids, liberated by lipoprotein lipase, influenced by the degree of unsaturation or the position of the double bonds in the molecule (44,45). This does not rule out, however, the possibility that fatty acids in the 2-position of monoglycerides have a different metabolic fate than those in the 1,3 position. The data of Chen et al. (43,44) also suggests that the positional specificity of lipoprotein lipase for the 1 and 3 position of triglyceride molecules is not influenced by the type of fatty acid occupying the 1 or 3 position.

Data from Chen et al. (42–45) and El Boustani et al. (47), as well as other investigators (124,164,166–169), indicate that n-3 fatty acids are not randomly distributed in the lipid classes, once hepatic and cellular metabolic processes take over from the absorption and initial transport phenomena. Indeed, the extensive literature on the n-3 fatty acid composition of platelets supports this contention (137,170–175).

The reported distribution of absorbed n-3 fatty acids indicates a slight enrichment of n-3 fatty acids in the phospholipid and mono- and diglyceride fractions of chylomicrons and a concomitant decrease in the triglyceride fraction, when compared to oleic acid in rats (45). In addition, there does not seem to be any major differences between the redistribution of eicosapentaenoic acid and oleic acid to the serum lipoproteins from chylomicrons after four hours in the rat (44). This is a somewhat

unexpected finding, because the distribution of triglyceride, cholesteryl ester and phospholipids is quite different among the various lipoprotein classes (176). Hence, one would expect oleic acid and eicosapentaenoic acid to be distributed in the IDL, LDL and HDL fraction with respect to the lipid class distribution, given the known preference for polyunsaturated fatty acids to be found in the phospholipids and for oleic acid to be incorporated into triglycerides (176,177). However, phospholipids in serum lipoproteins are mainly phosphatidylcholine, lysophosphatidylcholine and sphingomyelin (178). With the known preference of n-3 fatty acids for phosphatidylethanolamine and a lesser preference for phosphatidylcholine, this could simply reflect the phospholipid class composition in the lipoprotein fractions (178). Serum lipoproteins revert to a characteristic fatty acid composition in periods when no dietary fat is being absorbed (179). This is influenced only slightly by the fatty acids in the tissue body pools (180). Indeed, even in long-term feeding studies, the serum fatty acid composition is remarkably constant and characteristic of the species (181–184) during fasting periods, despite large and enduring changes in the tissue fatty acid composition. This suggests that there is a strong genetic determinate of the fatty acid composition of serum lipoprotein and probably reflects the character of the enzymes in the fatty acid metabolic pools in the liver of each species. These enzyme systems were probably evolved to adapt to the usual pattern of intake of dietary fat over evolutionary time spans and, therefore, should be unresponsive to temporary dietary alteration.

If this supposition is true, it could explain many of the purported pharmacological and pathological effects of dietary fatty acids. Unlike proteins or carbohydrates that are digested to common precursor molecules, i.e., simple amino acids or monosaccharides, and absorbed as such, each fatty acid retains its unique chemical properties during the digestion process. Thus, the source of protein or carbohydrate matters little to an organism, assuming the protein contains all the essential amino acids, but an unusual source of fat in the diet may provide very different fatty acids. These fatty acids may profoundly alter the physiology of the organism, if such fat is very different in its fatty acid composition from the animal's usual source of dietary fat.

It is probable that both the amount of fat calories and the type of fatty acids in the diet are responsible for many of the adverse metabolic changes found in fatty-acid-feeding studies. The main reason for this is the genetic basis of intermediary metabolism, which itself is a consequence of evolutionary adaptation to available food supplies (39). Omnivores have obvious evolutionary advantages over those less flexible in their dietary habits, but even omnivores have established dietary patterns, deviations from which can have adverse consequences on health. It may take an equivalent evolutionary time span to adapt to such dietary changes, if they are to become permanent.

Thus, incorporation of exogenous fatty acids into lipid classes, the uptake of fatty acids and lipids by organ systems in the body, and the composition and resident time of fatty acids in body pools are all probably under genetic control. Environmental factors only have a limited influence on these processes.

In most mammalian systems, linolenic acid is strongly discriminated against (1). It does not accumulate in tissue when normal diets are consumed (181,185) and is rapidly eliminated from tissue pools after feeding diets containing large amounts of this compound (Nelson, unpublished observations). Only in a few terrestrial plants (in contrast to marine flora which produce abundant amounts of n-3 fatty acids) does this particular fatty acid become a major component of the total fatty acids present (1). Yet, it could hardly be that it is too unsaturated or not susceptible to further metabolic manipulation, as many more unsaturated fatty acids are common in most tissue (186) and linolenic acid is converted slowly to eicosapentaenoic and docosahexaenoic acids in mammalian tissues (156,187–189). Obviously, the evolutionary pattern in animals has been to eliminate use of linolenic acid in favor of linoleic acid. Although readily converted to arachidonic acid and, in some cases, n-6 docosapentaenoic acid, linoleic acid itself is a major fatty acid of most body tissue pools (186). The evolutionary basis for discrimination against linolenic acid is an interesting but unexplained phenomenon, the elucidation of which awaits a greater understanding of lipid metabolism and adaptation processes.

SUMMARY AND CONCLUSIONS

Most of the data on the absorption and transport of n-3 polyunsaturated fatty acids has been obtained through studies with experimental animals. Some caution should, therefore, be exercised in extrapolating such results to humans. However, fat absorption studies are difficult to conduct in humans, so the animal experiments will remain the foundation of our knowledge in this area for some time.

The major unresolved issues regarding n-3 fatty acid absorption and transport appear to fall into two categories: 1) Are long-chain, n-3 polyunsaturated fatty acids, attached to the 2 position of triglycerides, metabolized similarly, and are they as functionally effective as those in the 1-3 position? 2) Are methyl or ethyl ester acceptable vehicles for the administration of n-3 polyunsaturated fatty acids?

It is clear that as far as absorption is concerned, long-chain n-3 polyunsaturated fatty acids in triglycerides, with the possible exception of linolenic acid, are not absorbed differentially. The absorption of linolenic acid needs further study. All 2-monoglycerides, regardless of which specific fatty acid is incorporated into the molecule, are equally well-absorbed, but their fate once reesterified to triglycerides in the enterocyte also needs further study.

The use of methyl or ethyl esters of n-3 fatty acid to deliver fatty acids to tissue would appear feasible, although it is clear that ethyl esters are poorly hydrolyzed by pancreatic lipase. Ethyl esters, not hydrolyzed in the intestinal lumen, may be carried farther in the intestinal tract to be hydrolyzed by the intestinal flora. It is possible that some ethyl esters of fatty acids may enter the enterocyte unhydrolyzed (47). They must then be rapidly hydrolyzed or transesterified in the cell, because none can be found in human circulation, after ingestion of ethyl ester fatty acids (114,121).

Recent evidence shows that plasma phospholipids and cholesteryl esters respond to EPA as the ethyl esters and to the same extent as from other administered forms

(123). This suggests that absorption is total, but may reflect EPA entering two lipid pools, one in chylomicron form and one possibly through the portal vein. The fate of the ethanol moiety is unknown, but presumedly it is converted to ethyl alcohol.

An interesting, but unstudied, facet of n-3 fatty acid digestion is the composition and fate of bile lecithin. The amount of exogenous phospholipid consumed is relatively small on a daily basis, estimated to be 1-2 g a day for humans (69,70). Bile, however, contributes about 20 g of phospholipid per day (63,69,70) and almost all of this is reabsorbed as free fatty acid and lysolecithin. Bile lecithin has a characteristic fatty acid composition (63,190), but the composition can be influenced by diet (191). In an acute feeding study, the fatty acid composition of the bile will not be influenced by the exogenous fatty acids, but in long-term fatty acid feeding studies, the bile lecithin fatty acid composition will change to reflect the dietary fatty acid composition to some degree. This, in turn, may influence the absorption of the dietary fat and, perhaps, cholesterol absorption.

Other major areas that need additional investigation are: 1) The lipolysis of triglycerides containing n-3 fatty acids by the lipases of the digestive tract—it is known that such triglycerides are relatively resistant to porcine pancreatic lipase (84,85,95-97) in vitro, but this phenomenon has been investigated only indirectly in vivo (43,98, 192); 2) the influence of dietary fatty acids on the distribution of lipoproteins in serum—there is preliminary evidence that n-3 fatty acids may raise LDL levels compared with n-6 fatty acids (8,193); and 3) the partitioning of n-3 fatty acids in the tissues. The specific metabolism of linolenic acid needs more investigation. There is no assurance that feeding linolenic acid will produce the same results as feeding eicosapentaenoic acid or docosahexaenoic acid. Furthermore, little or nothing is known about the metabolism or physiological action of n-3-series docosapentaenoic acid. It also would be interesting to determine how various dietary n-3 fatty acids affect intestinal and liver synthesis of apolipoproteins.

In summary, n-3 fatty acids are digested and absorbed in the mammalian digestive tract in ways similar to other saturated and unsaturated fatty acids. One notable difference in their digestion, however, is their resistance to lipolysis by pancreatic lipase, but this appears to effect more their rate of absorption into the circulation rather than the amount ultimately absorbed. They are incorporated into the chylomicrons and VLDL in the enterocyte. Chylomicrons containing n-3 fatty acids are appropriate substrate for lipoprotein lipase. These fatty acids are taken up by the liver and incorporated into all lipoprotein classes for transport to peripheral tissues where they are incorporated into the tissue fatty acid pools.

The methyl and ethyl esters of n-3 fatty acids are less readily hydrolysed by pancreatic lipase; hence, their absorption is slower when fed in this form (47,99), but may be equally complete. There are no balance studies where fecal excretion was measured comparing EPA or DHA ethyl ester absorption with that of triglyceride or free fatty acids.

Evidence has appeared recently (193) to suggest that, like saturated fatty acids, n-3 fatty acids can raise LDL levels which n-6 fatty acids depress, but more research is needed to confirm this result. However, if the observation proves to be true, then interesting questions are raised, in view of the epidemiological data suggesting that fish consumption reduces cardiovascular disease risk, concerning the mechanism by which n-3 fatty acids reduce the risk of cardiovascular disease. It should be noted, however, that the current evidence suggesting that n-3 fatty acids raise serum cholesterol conflicts with older data of Peifer et al. (194,195) and others (196-199) indicating that n-3 fatty acids markedly depressed serum cholesterol level. Some of the confusion may result from species dependent phenomena or the cholesterol content of fish oil (200), which is nominally 5 to 10 mg/g, but in fish oil concentrates the cholesterol content may not be known, although recent label claims for capsule products usually indicate < 1 to 5 mg/g (48). Further investigations will be needed to resolve this important issue.

Overall, it is clear that the absorption of fatty acids from the diet is still a subject not thoroughly understood. A lot of work has been accumulated in the past thirty years illuminating fundamental pathways of fat absorption and transport, but the mechanisms underlying the absorption and transport of specific fatty acids, with respect to chain length and degree of unsaturation, remain unclear. Recently, molecular biology has contributed considerable data on the fatty acid binding proteins (FABP) of the enterocyte, but the actual role of the FABP in fatty acid absorption and chylomicron formation is unknown (201). It could be that FABP show a fatty acid specificity that provides the mechanism by which the asymmetric distribution of fatty acids in tissue lipid is achieved. Currently, however, this is simply interesting speculation.

In view of the uncertainties establishing the necessary therapeutic intake-level of longer-chain n-3 polyunsaturated fatty acids (202), it seems appropriate to direct additional research toward a better understanding of the overall processes by which fat is digested, absorbed and transported.

REFERENCES

1. Tinoco, J. (1982) *Prog. Lipid Res. 21,* 1-45.
2. Goodnight, S.H., Harris, W.S., Connor, W.E., and Illingworth, D.R. (1982) *Arteriosclerosis 2,* 87-113.
3. Herold, P.M., and Kinsella, J.E. (1986) *Am. J. Clin. Nutr. 43,* 566-598.
4. Dyerberg, J., and Jorgensen, K.A. (1982) *Prog. Lipid Res. 21,* 255-269.
5. Bang, H.O., and Dyerberg, J. (1972) *Acta Med. Scand. 192,* 85-94.
6. Dyerberg, J., Bang, H.O., and Hjorne, N. (1974) *Am. J. Clin. Nutr. 28,* 958-966.
7. Dyerberg, J. (1981) *Phil. Trans. R. Soc. Lond. B 294,* 373-381.
8. Thiery, J., and Seidel, D. (1987) *Atherosclerosis 63,* 53-56.
9. Chamberlain, J.G., and Belton, C. (1987) *Atherosclerosis 68,* 95-103.
10. Dyerberg, J., and Bang, H.O. (1979) *Lancet 2,* 433-435.
11. Sanders, T.A.B., Vickers, M., and Haines, A.P. (1981) *Clin. Sci. 61,* 317-324.
12. Thorngren, M., and Gustafson, A. (1981) *Lancet ii,* 1190-1193.
13. Ahmed, A.A., and Holub, B.J. (1984) *Lipids 19,* 617-624.
14. Bruckner, G.G., Lokesh, B., German, B., and Kinsella, J.E. (1984) *Thromb. Res. 34,* 479-497.
15. Renaud, S., Martin, J.L., and Thevenon, C. (1987) in *Proceedings of the AOCS Short Course on Polyunsaturated Fatty Acids and Eicosanoids* (W.E.M. Lands, ed.) pp. 56-61, American Oil Chemists' Society, Champaign, IL.

16. Agradi, E., Tremoli, E., Colombo, C., and Galli, C. (1978) *Prostaglandins 16,* 973-984.
17. Knapp, H.R., Reilly, I.A.G., Alessandrini, P., and Fitzgerald, G.A. (1986) *N. Eng. J. Med. 314,* 937-942.
18. Nagakawa, Y., Orimo, H., Harasawa, M., Morita, I., Yashiro, K., and Murota, S. (1983) *Atherosclerosis 47,* 71-75.
19. Mortensen, J.Z., Schmidt, E.B., Nielsen, A.H., and Dyerberg, J. (1983) *Thromb. Haemostas. 50,* 543-546.
20. Driss, F., Vericel, E., Lagarde, M., Dechavanne, M., and Darcet, Ph. (1984) *Thromb. Res. 36,* 389-396.
21. Brox, B.H., Killie, J.E., Gunnes, S., and Norday, A. (1981) *Tromb. Haemostas. 46,* 604-611.
22. Salo, M.K. Vartiainen, E., Puska, P., and Nikkari, T. (1985) *Thromb. Haemostas. 54,* 563-569.
23. Bruckner, G., Webb, P., Greenwell, L., Chow, C., and Richardson, D. (1987) *Atherosclerosis 66,* 237-245.
24. Hamazaki, T., Kobayashi, S., Urahaze, M., Yano, S., and Fujita, T. (1985) *Biorheology 22,* 221-226.
25. Kobayashi, S., Hamazaki, T., Hirai, A., Terano, T., Tamura, Y., Kanakubo, Y., Yoshida, S., Fujita, T., and Kumagai, A. (1985) *Clin. Hemorheology 5,* 493-505.
26. Hornstra, G., and Rand, M.L. (1986) *Prog. Lipid Res. 25,* 637-638.
27. Abeywardena, M.Y., McLennan, P.L., and Charnock, J.S. (1987) in *Proceedings of the AOCS Short Course on Polyunsaturated Fatty Acids and Eicosanoids* (W.E.M. Lands, ed.) pp. 62-65, American Oil Chemists' Society, Champaign, IL.
28. Kanazawa, A., Teshima, S.I., Tokiwa, S., and Imatanaka, N. (1984) *Mem. Fac. Fisch. Kagoshima Univ. 33,* 73-78.
29. Weiner, B.H., Ockene, I.S., Levine, P.H., Cuenoud, H.F., Fisher, M., Johnson, B.F., Daoud, A.S., Jarmolych, J., Johnson, M.H., Natale, A., Vandreuil, C., and Hoogasian, J.J. (1986) *N. Eng. J. Med. 315,* 841-846.
30. Davis, H.R., Bridenstine, R.T., Vesselinovitch, D., and Wissler, R.W. (1987) *Arteriosclerosis 7,* 441-449.
31. Kromhout, D., Bosschieter, E.B., and de Lezzene-Coulander, C. (1985) *N. Eng. J. Med. 312,* 1205-1209.
32. Storlien, L.H., Kraegen, E.W., Chisholm, D.J., Ford, G.L., Bruce, D.G., and Pascoe, W.S. (1987) *Science 237,* 885-888.
33. Holman, R.T., Johnson, S.B., and Hatch, T.F. (1982) *Am. J. Clin. Nutr. 35,* 617-623.
34. Stein, T.P., Marino, P.L., Harner, R.N., Schluter, M.D., Leskiw, M.J., and Black, S. (1983) *J. Am. Coll. Nutr. 2,* 241-247.
35. Neuringer, M., Connor, W.E., Van Petten, C., and Barstad, L. (1984) *J. Clin. Invest. 73,* 272-276.
36. Zoller, N. (1986) *Prog. Lipid Res. 25,* 177-180.
37. Yamanaka, W.K., Clemens, G.W., and Hutchinson, M.L. (1981) *Prog. Lipid Res. 19,* 187-215.
38. Bjerve, K.S., Mostad, I.L., and Thoresen, L. (1987) *Am. J. Clin. Nutr. 45,* 66-77.
39. Lands, W.E.M. (1986) *Fish and Human Health,* pp. 15-19, Academic Press, Orlando, FL.
40. Borgstrom, B. (1976) in *Lipid Absorption: Biochemical and Clinical Aspects* (K. Rommel, H. Goebell, and R. Boehmer, eds.) pp. 65-72, University Park Press, Baltimore, MD.
41. Patton, J.S. (1981) in *Physiology of the Gastro-intestinal Tract* (L.R. Johnson, ed.) pp. 1123-46, Raven Press, NY.
42. Chen, I.S., Subramanian, S., Cassidy, M.M., Sheppard, A.J. and Vahouny, G.V. (1985) *J. Nutr. 115,* 219-225.
43. Chen, I.S., Hotta, S.S., Ikeda, I., Cassidy, M.M., Sheppard, A.J., and Vahouny, G.V. (1987) *J. Nutr. 117,* 1676-1680.
44. Chen, I.S., Sheppard, A.J., Cassidy, M.M., and Vahouny, G.V. (1987) *Atherosclerosis 65,* 193-198.
45. Chen, I.S., Le, T., Subramanian, S., Cassidy, M.M., Sheppard, A.J., and Vahouny, G.V. (1987) *Lipids 22,* 318-321.
46. Ackman, R.G. (1988) *Atherosclerosis 70,* 171-173.
47. El Boustani, S., Colette, C., Monnier, L., Descomps, B., Crastes de Paulet, A., and Mendy, F. (1987) *Lipids 22,* 711-714.
48. Ackman, R.G. (1988) *Chem. Ind.,* pp. 139-145.
49. Naughton, J.M. (1981) *Int. J. Biochem. 13,* 21-32.
50. Siguel, E.N., and Maclure, M. (1987) *Metabolism 36,* 664-669.
51. Kinsella, J.E. (1987) *Seafoods and Fish Oils in Human Health and Disease,* Marcel Dekker, Inc., New York, NY.
52. Lands, W.E.B., ed. (1987) *Proceedings of the AOCS Short Course on Polyunsaturated Fatty Acids and Eicosanoids,* American Oil Chemists' Society, Champaign, IL.
53. Simopoulous, A.P., Kifer, P.R., and Martin, R.E., eds. (1986) *Health Effects of Polyunsaturated Fatty Acids in Seafoods,* Academic Press, Orlando, FL.
54. Small, D.M. (1986) in *Handbook of Lipid Research,* Vol. 4 (D.J. Hanahan, ed.) pp. 43-96, Plenum Press, New York, NY.
55. Bisagaier, C., and Glickman, R.M. (1976) *Ann. Rev. Physiol. 45,* 625-636.
56. Muller, D.P.R., McCullum, J.P.K., Trompeter, R.S., and Harries, J.T. (1975) *Gut 16,* 838-847.
57. Moreau, H., Gargouri, Y., Bernadal, A., Pieroni, G., and Verger, R. (1988) *Rev. Franc. Corps Gras 35,* 169-176.
58. DeNigris, S.J., Hamosh, M., Kasbekar, D.K., Lee, C.L., and Hamosh, P. (1988) *Biochim. Biophys. Acta 959,* 38-45.
59. Jandacek, R.J., Whiteside, J.A., Holcombe, B.N., Volpenhein, R.A., and Taulbee, J.D. (1987) *Am. J. Clin. Nutr. 45,* 940-945.
60. Hyun, S.A., Vahouny, G.V., and Treadwell, C.R. (1967) *Biochim. Biophys. Acta 137,* 296-305.
61. Vallot, A., Bernard, A., and Carlier, H. (1985) *Comp. Biochem. Physiol. 82A,* 693-699.
62. Jackson, M.J. (1974) in *Biomembranes,* Vol. 4B (Smyth, D.H., ed.) pp. 673-709, Plenum Press, New York, NY.
63. Tso, P. (1985) *Adv. Lipid Res. 21,* 143-186.
64. Borgstrom, B. (1974) in *Biomembranes,* Vol. 4B (Smyth, D.H., ed.) pp. 555-620, Plenum Press, New York, NY.
65. Tidwell, H.C., Pope, J.L., Askins, R.E., and MacPherson, J.C. (1963) *Biochem. Probl. Lipids 1,* 219-223.
66. Nilsson, A. (1969) *Biochim. Biophys. Acta 187,* 113-121.
67. MacDonald, G.B., Saunders, D.R., Weidman, M., and Fisher, L. (1980) *Am. J. Physiol. 239,* G 141-150.
68. Surawicz, C.M., Saunders, D.R., Sillery, J., and Rubin, C.E. (1981) *Am. J. Physiol. 240,* G 157-162.
69. Carey, M.C., Small, D.M., and Bliss, C.M. (1983) *Ann. Rev. Physiol. 45,* 651-678.
70. Borgstrom, B. (1977) *Int. Rev. of Physiol. Gastrointest. Physiol. II 12,* 305-323.
71. Borgstrom, B. (1977) in *Cholesterol Metabolism and Lipolytic Enzymes* (J. Polonovski, ed.) pp. 173-182, Masson Publishing USA, New York, NY.
72. Brown, J.L., and Johnston, J.M. (1964) *Biochim. Biophys. Acta 84,* 264-274.
73. Simmonds, W.J. (1972) in *Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism* (G.J. Nelson, ed.) pp. 705-743, Wiley-Interscience, New York, NY.
74. Havel, R.J. (1985) *Arteriosclerosis 5,* 569-590.
75. Kane, J.P. (1983) *Ann. Rev. Physiol. 45,* 637-650.
76. Kane, J.P., Chen, G.C., Hamilton, R.L., Hardman, D.A., Malloy, M.J., and Havel, R.J. (1983) *Arteriosclerosis 3,* 47-56.
77. Fielding, C.J., and Fielding, P.E. (1977) in *Cholesterol Metabolism and Lipolytic Enzymes* (J. Polonovski, ed.) pp. 165-172, Masson Publishing USA, New York, NY.
78. Scow, R.O., Blanchette, J. and Smith, L.C. (1977) in *Cholesterol Metabolism and Lipolytic Enzymes* (J. Polonovski, ed.) pp. 143-164, Masson Publishing USA, New York, NY.
79. Jones, A.L., Hradek, G.T., Hornick, C., Renaud, G., Windler, E.E., and Havel, R.J. (1984) *J. Lipid Res. 25,* 1151-1158.
80. Hui, D.Y., Innerarity, T.L., and Mahley, R.W. (1981) *J. Biol. Chem. 256,* 5646-5655.
81. Havel, R.J. (1982) *Med. Clin. North Am. 66,* 319-333.
82. Nilsson-Ehle, P., Egelrud, T., Belfrage, P., Olivecrona, T., and Borgstrom, B. (1973) *J. Biol. Chem. 248,* 6734-6737.
83. Morley, N.H., Kuksis, A., Buchnea, D., and Myher, J.J. (1975) *J. Biol. Chem. 250,* 3414-3418.
84. Brockerhoff, H., Hoyle, R.J., and Huang, P.C. (1966) *Can. J. Biochem. 44,* 1519-1525.
85. Bottino, N.R., Vandenburg, G.A., and Reiser, R. (1967) *Lipids 2,* 489-493.
86. Scow, R.O., and Egelrud, T. (1976) *Biochim. Biophys. Acta 431,* 538-549.
87. Bergman, E.N., Havel, R.J., Wolfe, B.M., and Bohmer, T. (1971) *J. Clin. Invest. 50,* 1831-1839.
88. Nicoll, A., Miller, N.E., and Lewis, B. (1980) in *Advances in Lipid Research* (R. Paoletti and D. Kritchevsky, eds.) Vol. 17, pp. 53-106, Academic Press, New York.
89. Glomset, J.A., and Verdery, R.B. (1977) in *Cholesterol*

Metabolism and Lipolytic Enzymes (J. Polonovski, ed.) pp. 137-142, Masson Publishing USA, New York, NY.
90. Tall, A.R., and Small, D.M. (1980) in *Advances in Lipid Research* (R. Paoletti and D. Kritchevsky, eds.) Vol. 17, pp. 2-51, Academic Press, New York, NY.
91. Goldstein, J.L., Kita, T., and Brown, M.S. (1983) *N. Eng. J. Med. 309,* 288-296.
92. Sodhi, H.S., Kudchodkar, J.B., and Mason, D.T. (1978) in *Advances in Lipid Research* (R. Paoletti and D. Kritchevsky, eds.) Vol. 17, pp. 107-153, Academic Press, New York, NY.
93. Noble, R.C. (1978) *Prog. Lipid Res. 17,* 55-91.
94. Noble, R.C. (1980) *Prog. Lipid Res. 18,* 179-216.
95. Brockerhoff, H. (1965) *Arch. Biochem. Biophys. 110,* 586-592.
96. Yurkowski, M., and Brockerhoff, H. (1966) *Biochim. Biophys. Acta 125,* 55-59.
97. Brockerhoff, H. (1970) *Biochim. Biophys. Acta 212,* 92-101.
98. Harris, W.S., and Connor, W.E. (1980) *Trans. Assoc. Am. Physicians 93,* 148-155.
99. Lawson, L.D., and Hughes, B.G. (1988) *Biochem. Biophys. Res. Commun. 152,* 328-335.
100. Wu, A.L., Clark, S.B., and Holt, P.R. (1980) *Am. J. Clin. Nutr. 33,* 582-589.
101. Carroll, K.K. (1958) *J. Nutr. 64,* 399-410.
102. Feldman, E.B., Russell, B.S., Schnare, F.H., Miles, B.C., and Doyle, E.A. (1979) *J. Nutr. 109,* 2226-2236.
103. Thomson, A.B.R. (1980) *J. Lipid Res. 21,* 1097-1107.
104. Soichura, M., Asakura, H., Nagata, H., Marishita, T., Watanabe, M., and Tsuchiya, M. (1981) *Bibl. Anat. 20,* 289-295.
105. Jones, P.J.H., Pencharz, P.B., and Clandinin, M.T. (1985) *Am. J. Clin. Nutr. 42,* 769-777.
106. Ockner, R.K., Pittman, J.P., and Yager, J.L. (1972) *Gastroenterology 62,* 981-992.
107. Mattil, K.F., and Higgins, J.W. (1945) *J. Nutr. 29,* 255-260.
108. Mattson, F.H. (1959) *J. Nutr. 69,* 338-342.
109. Tomarelli, R.M., Meyer, B.J., Weaber, J.R., and Bernhart, F.W. (1968) *J. Nutr. 95,* 583-590.
110. Filer, F.J., Jr., Mattson, F.H., and Fomon, S.J. (1969) *J. Nutr. 99,* 293-298.
111. Bonanome, A., and Grundy, S.M. (1988) *New Eng. J. Med. 318,* 1244-1248.
112. Vahouny, G.V. (1982) in *Dietary Fiber in Health and Disease* (G.V. Vahouny and D. Kritchevsky, eds.) pp. 203-227, Plenum Press, New York, NY.
113. Alfin-Slater, R.B., Morris, R.S., Hansen, H., and Proctor, J.F. (1965) *J. Nutr. 87,* 168-172.
114. Terano, T., Hirai, A., Hamazaki, T., Kobayashi, S., Fujita, T., Tamura, Y., and Kumagai, A. (1983) *Atherosclerosis 46,* 321-331.
115. Hamazaki, T., Hirai, A., Terano, T., Sajiki, J., Kondo, S., Fujita, T., Tamura, Y., and Kumagai, A. (1982) *Prostaglandins 23,* 557-568.
116. Bjerve, K.S., Fischer, S., and Alme, K. (1987) *Am. J. Clin. Nutr. 46,* 570-576.
117. Yoshimura, T., Ito, M., Matsui, K., and Fujisaki, S. (1986) *Prostaglandins 32,* 179-188.
118. Holme, S., Brox, J.H., Krane, H., and Nordoy, A. (1984) *Thromb. Haemostas. 51,* 32-36.
119. Croset, M., and Lagarde, M. (1986) *Thromb. Haemostas. 56,* 57-62.
120. Hajarine, M., and Lagarde, M. (1986) *Biochim. Biophys. Acta 877,* 299-304.
121. Nagakawa, Y., Orimo, H., Murota, S., and Murita, I. (1982) *Domyaku Koda 10,* 631-640.
122. Velardo, B., Lagarde, M., Guichardant, M., Dechavanne, M., Beylot, M., Sautot, G., and Berthezene, F. (1982) *Thromb. Haemostas. 48,* 344.
123. Hamazaki, T., Urakaze, M., Makuta, M., Ozawa, A., Soda, Y., Tatsumi, H., Yano, S., and Kumagai, A. (1987) *Lipids 22,* 994-998.
124. Philbrick, D.J., Mahadevappa, V.G., Ackman, R.G., and Holub, B.J. (1987) *J. Nutr. 117,* 1663-1670.
125. Shimizu, S., and Yoshihara, S. (1977) *Agric. Biol. Chem. 41,* 1525-1527.
126. Boidron, J.N., Lefebvre, A., Riboulet, J.M., and Ribereau-Gayon, P. (1984) *Sci. Aliments. 4,* 609-616.
127. Lange, L.G., Bergman, S.R., and Sobel, B.E. (1981) *J. Biol. Chem. 256,* 12968-12973.
128. Lange, L.G., and Sobel, B.E. (1983) *J. Clin. Invest. 72,* 724-731.
129. Vahouny, G., Chen, I.S., Satchithanandam, S., Cassidy, M.M. and Sheppard, A.J. (1985) in *Prostaglandins, Leukotrienes and Lipoxins* (J.M. Bailey, ed.) pp. 393-403, Plenum Press, New York, NY.
130. Ricci, S., Patoia, L., Berrettini, M., Binaglia, L., Scarcella, M.G., Bucaneve, G., Vecchini, A., Carloni, I., Agostini, L., Parise, P., and Del Favero, A. (1987) *Stroke 18,* 575-578.
131. Bruckner, C.G., Lokesh, B., German, B., and Kinsella, J.E. (1984) *Thrombosis Res. 34,* 479-497.
132. Takeuchi, H., Ito, A., Murase, T., Nagai, M., and Muramatsu, K. (1984) *Agric. Biol. Chem. 48,* 2125-2127.
133. Horrobin, D.F., Huang, Y.S., Cunnane, S.C., and Manku, M.S. (1984) *Lipids 19,* 806-811.
134. Fragiskos, B., Chan, A.C., and Choy, P.C. (1986) *Ann. Nutr. Metabol. 30,* 331-334.
135. Urakaze, M., Hamazaki, T., Soda, Y., Miyamoto, A., Ibuki, F., Yano, S., and Kumagai, A. (1986) *Thrombosis Res. 44,* 673-682.
136. Carlson, S.E., Rhodes, P.G., and Ferguson, M.G. (1986) *Am. J. Clin. Nutr. 44,* 798-804.
137. Siess, W., Roth, P., Scherer, B., Kurzman, T., Bohlig, F., and Weber, P.C. (1980) *Lancet i,* 441-444.
138. Fischer, S., and Weber, P.C. (1983) *Biochem. Biophys. Res. Commun. 116,* 1091-1099.
139. Brindley, D.N. (1974) in *Biomembranes* (Smyth, D.H., ed.) Vol. 4B, pp. 621-671, Plenum Press, New York, NY.
140. Schiller, C.M., David, J.S.K., and Johnston, J.M. (1970) *Biochim. Biophys. Acta 210,* 489-492.
141. Rao, G.A., and Johnston, J.M. (1966) *Biochim. Biophys. Acta 125,* 465-473.
142. Polheim, D., David, J.S.K., Schultz, F.M., Wylie, M.B., and Johnston, J.M. (1973) *J. Lipid Res. 14,* 415-421.
143. Schaefer, E.J., Eisenberg, S., and Levy, R.I. (1978) *J. Lipid Res. 19,* 667-687.
144. Green, P.H.R., and Glickman, R.M. (1981) *J. Lipid Res. 22,* 1153-1173.
145. Morre, D.J. (1977) *Cell. Surf. Rev. 4,* 1-83.
146. Aviram, M., Brox, J., and Nordoy, A. (1986) *Ann. Nutr. Metab. 30,* 143-148.
147. Van Gent, C.M., Luten, J.B., Bronsgeest-Schoute, H.C., and Ruiter, A. (1979) *Lancet ii,* 1249-1250.
148. Saynor, R., Gillott, T., Dolye, T., Allen, D., Field, P., and Scott, M. (1986) *Prog. Lipid Res. 25,* 211-217.
149. Kobatake, Y., Kuroda, K., Jinnouchi, H., Nishide, E., and Innami, S. (1984) *J. Nutr. Sci. Vitaminol. 30,* 357-372.
150. Sanders, T.A.B., Sullivan, D.R., Reeve, J. and Thompson, G.R. (1985) *Arteriosclerosis 5,* 459-465.
151. Anding, R.H., and Hwang, D.H. (1986) *Lipids 21,* 697-701.
152. Nossen, J.O., Rustan, A.C., Gloppestad, S.H., Malbakken, S., and Drevon, C.A. (1986) *Biochim. Biophys. Acta 879,* 56-65.
153. Daggy, B., Arost, C., and Bensadoun, A. (1987) *Biochim. Biophys. Acta 920,* 293-300.
154. Wong, S., and Nestel, P.J. (1987) *Atherosclerosis 64,* 139-146.
155. Puppione, D.L., Corash, L., Kunitake, D.T., Smith, D.L., and Costa, D.P. (1987) in *Polyunsaturated Fatty Acids and Eicosanoids* (W.E.M. Lands, ed.) pp. 352-357, American Oil Chemists' Society, Champaign, IL.
156. O'Dea, K., Steel, M., Naughton, J., Sinclair, A., Hopkins, G., Angus, J., He, G.-W., Niall, M., and Martin, T.J. (1988) *Lipids 23,* 234-341.
157. Dervichian, D.G. (1954) in *Progress in the Chemistry of Fats and Other Lipids* (R.T. Holman, W.O. Lundberg and T. Malkin, eds.) Vol. 2, pp. 193-243, Pergamon Press, New York, NY.
158. Bronsgeest-Schoute, H.C., Van Gent, C.M., Luten, J.B., and Ruiter, A. (1981) *Am. J. Clin. Nutr. 34,* 1752-1757.
159. Holub, B.J., Bakker, D.J., and Skeaff, C.M. (1987) *Atherosclerosis 66,* 11-18.
160. Savary, P., and Constantin, M.J. (1987) *Biochim. Biophys. Acta 144,* 549-555.
161. Weiner, T.W., and Sprecher, H. (1984) *Biochim. Biophys. Acta 792,* 293-303.
162. Swanson, J.E., Black, J.M., and Kinsella, J.E. (1987) *J. Nutr. 117,* 824-832.

163. Iritani, N., and Narita, R. (1984) *Biochim. Biophys. Acta 793,* 441-447.
164. Parks, J.S., and Bullock, B.C. (1987) *J. Lipid Res. 28,* 173-182.
165. Parks, J.S., Martin, J.A., Sonbert, B.L., and Bullock, B.C. (1987) *Arteriosclerosis 7,* 71-79.
166. Mahadevappa, V.G., and Holub, B.J. (1982) *Biochim. Biophys. Acta 713,* 73-79.
167. Popp-Snijders, C., Schouten, J.A., De Jong, A.P., and van der Veen, E.A. (1984) *Scand. J. Clin. Lab. Invest. 44,* 39-46.
168. Popp-Snijders, C., Schouten, J.A., Van Blitterswijk, W.J., and van der Veen, E.A. (1986) *Biochim. Biophys. Acta 854,* 31-37.
169. Weaver, B.J., and Holub, B.J. (1985) *Lipids 20,* 773-777.
170. Goodnight, S.H., Harris, W.S., and Conner, W.E. (1981) *Blood 58,* 880-884.
171. Mori, T.A., Codde, J.P., Vandongen, R., and Beilin, L.J. (1987) *Lipids 22,* 744-750.
172. von Schacky, C., and Weber, P.C. (1985) *J. Clin. Invest. 76,* 2446-2450.
173. von Schacky, C., Fischer, S., and Weber, P.C. (1985) *J. Clin. Invest. 76,* 1626-1631.
174. von Schacky, C., Siess, W., Fischer, S., and Weber, P.C. (1985) *J. Lipid Res. 26,* 457-464.
175. Galloway, J.H., Cartwright, I.J., Woodcock, B.E., Greaves, M., Russell, R.G., and Preston, F.E. (1985) *Clin. Sci. 68,* 449-454.
176. Iritani, N., Mizoguchi, H., and Kitaya, M. (1987) *Lipids 22,* 45-50.
177. Iritani, N., Ikeda, Y., and Kajitani, H. (1984) *Biochim. Biophys. Acta 793,* 416-422.
178. Skipski, V.P. (1972) in *Blood Lipids: Quantitation, Composition and Metabolism* (G.J. Nelson, ed.) pp. 471-583, Wiley-Interscience, New York, NY.
179. Moore, R.A., Oppert, S., Eaton, P., and Mann, J.I. (1977) *Clin. Endocrinol. 7,* 143-149.
180. Wood, D.A., Butler, S., Macintyre, C., Reimersma, R.A., Thomson, M., Elton, R.A., and Oliver, M.F. (1987) *Lancet ii,* 177-183.
181. Moilanen, T., Nikkari, T., Rasanen, L., Viikari, J., Akerblom, H.K., Ahola, M., Dahl, M., Lahde, P-L., Pesonen, E., Pietikainen, M., Seppanen, A., Suoninen, P., and Uhari, M. (1983) *Atherosclerosis 48,* 49-56.
182. Nikkari, T., Salo, M., Maatela, J., and Aromaa, A. (1983) *Atherosclerosis 49,* 139-148.
183. Nikkari, T., Rasanen, L., Viikari, J., Akerblom, H.K., Vuori, I., Pyorala, K., Uhari, M., Dahl, M., Lahde, P.-L., Pesonen, E., and Suoninen, P. (1983) *Am. J. Clin. Nutr. 37,* 848-854.
184. Dougherty, R.M., Galli, C., Ferro-Luzzi, A., and Iacono, J.M. (1987) *Am. J. Clin. Nutr. 45,* 443-455.
185. Holmer, G., and Beare-Rogers, J.L. (1985) *Nutr. Res. 5,* 1011-1014.
186. Manku, M.S., Horrobin, D.F., Huang, Y-S., and Morse, N. (1983) *Lipids 18,* 906-908.
187. Spector, A.A., Kaduce, T.L., Figard, P.H., Norton, K.C., Hoak, J.C., and Czervionke, R.L. (1983) *J. Lipid Res. 24,* 1595-1604.
188. Tou, J-S. (1984) *Lipids 19,* 573-577.
189. Careaga, M.M., and Sprecher, H. (1984) *J. Biol. Chem. 259,* 14413-14417.
190. Balint, J.A., Beeler, D.A., Treble, D.H., and Spitzer, H.L. (1967) *J. Lipid Res. 8,* 486-493.
191. Tso, P., Balint, J.A., and Simmonds, W.J. (1977) *Gastroenterology 73,* 1362-1367.
192. Boyce, J., and Fordyce, F. (1987) *Human Nutr.: Applied Nutr. 41A,* 364-366.
193. Harris, W.S., Dujovne, C.A., Zucker, M.L., and Johnson, B.E. (1987) *Circulation 76,* Suppl. iv, p. 168.
194. Peifer, J.J., Jannsen, F., Ahn, P., Cox, W., and Lundberg, W.O. (1960) *Arch. Biochem. Biophys. 86,* 302-308.
195. Peifer, J.J., Lundberg, W.O., Ishio, S., and Warmanen, E. (1965) *Arch. Biochem. Biophys. 110,* 270-283.
196. Kobatake, Y., Hirahara, F., Innami, S., and Nishide, E. (1983) *J. Nutr. Sci. Vitaminol. 29,* 11-21.
197. Von Lossonczy, T.O., Ruiter, A., Bronsgeest-Schoute, H.C., van Gent, C.M., and Hermus, R.J.J. (1978) *Am. J. Clin. Nutr. 31,* 1340-1346.
198. Singer, P., Walfraud, J., Wirth, M., Voight, S., Nauman, E., Zimontkowski, S., Hajdu, I., and Goedicke, W. (1983) *Atherosclerosis 49,* 99-108.
199. Phillipson, B.E., Rothrock, D.W., Connor, W.E., Harris, W.S., and Illingworth, D.R. (1985) *N. Engl. J. Med. 312,* 1210-1219.
200. Bimbo, A.P. (1986) in *n-3 News I* (No. 3), p. 1, Department of Preventive Medicine, Harvard Medical School, Boston, MA.
201. Sweetser, D.A., Heuckeroth, R.O., and Gordon, J.I. (1987) *Ann. Rev. Nutr. 7,* 337-359.
202. Driss, F., and Darcet, Ph. (1988) *Rev. Franc. Corps Gras 35,* 7-11.

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ARTICLES

cis-9,10-Epoxystearic Acid in Human Leukocytes: Isolation and Quantitative Determination

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Racemic *cis*-9,10-epoxystearic acid was isolated from total lipids of human leukocytes. Identification of the epoxy acid was based mainly on gas liquid chromatographic-mass spectrometric analysis and on its chemical conversion into *threo*-9,10-dihydroxystearic acid. A mass spectrometric method for quantitative determination of *cis*-9,10-epoxystearic acid using the tetradeuterated compound as internal standard was developed. Using this method, nonstimulated human leukocytes were found to contain 5.1 ± 2.2 μg (SD) of *cis*-9,10-epoxystearic acid per 10^9 cells (n = 8). More than 90% of the epoxy acid occurred in its esterified form in leukocyte lipids.
Lipids 23, 1015–1018 (1988).

Recently, human leukocytes incubated with linoleic acid were reported to produce 9,10-epoxy-12-octadecenoic acid (1,2). The epoxy acid, which served as an uncoupling agent in mitochondrial respiration and caused relaxation of rat stomach smooth muscle, was named "leukotoxin." Biosynthesis of leukotoxin was enhanced in the presence of either calcium ion or the divalent ion ionophore, A 23187 (2). The presence of endogenous leukotoxin in unstimulated cells was also reported (2).

A study was initiated in our laboratory to examine the possible presence and formation of saturated epoxy acids in leukocytes. Such compounds might have easily escaped detection by the ultraviolet absorption method used in the previous work (1,2). The present paper is concerned with isolation and quantitative determination of one such saturated fatty acid epoxide in human leukocytes, i.e., *cis*-9,10-epoxystearic acid.

MATERIALS AND METHODS

Materials. Oleic and linoleic acids were purchased from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Linoleic acid was obtained from Amersham (Amersham, UK). Methyl *cis*-9,10-epoxystearate was prepared by epoxidation of methyl oleate by *m*-chloroperbenzoic acid (3). Methyl [1-^{14}C]*cis*-9,10-epoxystearate was obtained by monoepoxidation of [1-^{14}C]linoleic acid followed by catalytic hydrogenation and preparative thin layer chromatography (TLC) (methyl *cis*-9,10-epoxystearate, R_f = 0.37; methyl *cis*-12,13-epoxystearate, R_f = 0.43). The specific radioactivity of the specimen was $137*10^9$ cpm/mol.

threo-9,10-Dihydroxystearic acid was prepared by treatment of methyl *cis*-9,10-epoxystearate (5 mg) with formic acid (1 ml) at room temperature for 2 hr, followed by 1.2 ml of 0.17 M sodium hydroxide in 83% aqueous methanol at room temperature overnight. An aliquot of the dihydroxy acid was converted into the methyl ester-trimethylsilyl (Me_3Si) derivative and analyzed by gas chromatography-mass spectrometry (GC-MS). The mass spectrum showed prominent ions at *m/e* 332 (M-142; rearrangement with loss of $OHC-(CH_2)_7CH_3$), 259 ($Me_3SiO^+=CH-(CH_2)_7-COOCH_3$) and 215 ($Me_3SiO^+=CH-(CH_2)_7-CH_3$). This is in agreement with data published earlier (4).

erythro-9,10-Dihydroxystearic acid was prepared by osmium tetroxide hydroxylation of methyl oleate (5) followed by saponification. The mass spectrum of the methyl ester-Me_3Si derivative was virtually identical to that of the corresponding derivative of *threo*-9,10-dihydroxystearic acid (see above).

Human leukocytes in calcium-free phosphate buffered saline (PBS), pH 7.4, were prepared as previously described (6). The cell count was adjusted to 10^8/ml.

Methyl [9,10,12,13-$^2H_4$]cis-9,10-epoxystearate. Methyl [9,10,12,13-$^2H_4$]linoleate (10 mg) (7) was stirred with 50 μl of 100% hydrazinium hydroxide and 3 mg of cupric diacetate in 1 ml of methanol for 6 hr at room temperature. Gas liquid chromatographic (GLC) analysis of the product demonstrated the presence of methyl stearate, methyl 9- and 12-octadecenoates, as well as methyl linoleate. Epoxidation by *m*-chloroperbenzoic acid afforded a mixture of deuterated methyl 9,10- and 12,13-epoxystearates, as well as methyl 9,10,12,13-diepoxystearate. Pure methyl [9,10,12,13-$^2H_4$]*cis*-9,10-epoxystearate was obtained following preparative TLC. The isotopic composition was 7% trideuterated and 93% tetradeuterated molecules, as determined by mass spectrometry using the unlabeled compound as reference.

Methods. TLC was carried out with Merck Fertigplatten (Kieselgel 60, 0.25 mm). The solvent system used was ethyl acetate/hexane (15:85, v/v). Spots were visualized by spraying with 2,7-dichlorofluorescein and viewed under UV light.

Gas liquid chromatography was carried out with an F&M Biomedical Gas Chromatograph Model 402. The stationary phase used was 3% SE 30 on Supelcoport, except for analysis of menthoxycarbonyl derivatives, in which case the stationary phase was 5% QF-1 on Supelcoport. The column lengths were 1.8 m. Analysis of cyclic carbonate derivatives was performed with a Carlo Erba Strumentazione HRCG 5300 gas chromatograph. A 25-m fused silica capillary column of 007 methyl silicone was used. The column temperature was 250°C.

Mass spectrometry was carried out with an LKB 9000S instrument equipped with column of 1% SE 30 on Chromosorb W, except for the analysis of synthetic and biologically derived methyl 9,10-epoxystearate, in which case a column of 3% OV-210 on Supelcoport was used. The carrier gas was helium in all gas liquid chromatographs. In all mass spectras, the electron energy was 22.5 eV and the trap current 60 μA.

Abbreviations: PBS, phosphate buffered saline; GC-MS, gas chromatography-mass spectrometry; GLC, gas liquid chromatograph(ic,y); TLC, thin layer chromatography.

Radioactivity was determined with a Packard Tri-Carb series 4000 instrument. Methods for catalytic hydrogenation (8) and oxidative ozonolysis (9) and for preparation of Me_3Si (8), (−)-menthoxycarbonyl (9) and cyclic carbonate (5) derivatives were as previously described.

RESULTS

Isolation and identification of cis-*9,10-epoxystearic acid.* Suspensions of human leukocytes in calcium-free PBS (10^8 cells/ml) were added to 20 vol of chloroform/methanol (2:1, v/v). The mixture was kept at room temperature for 30 min. Precipitated protein was removed by filtration, and the clear filtrate was shaken with 0.9% saline (0.2 vol with respect to the volume of filtrate). The chloroform layer was taken to dryness, and the residue was treated with 10 ml of 0.5 M sodium hydroxide in 80% aqueous methanol at room temperature overnight. The solution was extracted with one portion of hexane and subsequently acidified and extracted with two portions of diethyl ether. The combined ether phases were washed until neutral and then taken to dryness. The residue was treated with diazomethane and subjected to preparative TLC using authentic methyl *cis*-9,10-epoxystearate as reference. The zone corresponding to the reference spot (R_f = 0.37) was scraped off and extracted with ethyl acetate. Material thus obtained was analyzed by GC–MS. A peak with a C value identical to that of authentic methyl *cis*-9,10-epoxystearate (C-22.0) appeared. The mass spectrum (Fig. 1) was identical to that of the reference compound and showed prominent ions inter alia at *m/e* 294 (M-18; loss of H_2O), 281 (M-31; loss of $^{\bullet}OCH_3$), 214 (M-98; elimination of $CH_2{=}CH{-}C_5H_{11}$ or its equivalent by cleavage of the 11–12 bond and rearrangement of one hydrogen atom), 199 (M-113; loss of $^{\bullet}(CH_2)_7{-}CH_3$) and 155 (M-157; loss of $^{\bullet}(CH_2)_7{-}COOCH_3$). This is in agreement with data published earlier (10,11). In addition, a minor peak appeared in the gas chromatogram. The retention time and mass spectrum of this material were identical to those of authentic methyl 9,10-epoxy-12-octadecenoate.

An aliquot of the epoxy ester isolated from leukocytes was treated with formic acid followed by sodium hydroxide. This treatment resulted in the formation of 9,10-dihydroxystearic acid as judged by GC–MS. The C value of the methyl ester-Me_3Si derivative was 21.4 (references, corresponding derivatives of *threo-* and *erythro*-9,10-dihydroxystearates, C-21.4 and C-21.5, respectively). The mass spectrum recorded on the Me_3Si derivative of the biologically derived dihydroxyester (Fig. 2) was identical to those of the Me_3Si derivatives of authentic methyl *threo-* and *erythro*-9,10-dihydroxystearates. Methyl 9,10-dihydroxystearate (≈20 μg) prepared from the biologically derived 9,10-epoxystearate was converted into the cyclic carbonate derivative (5) and analyzed by GLC using the cyclic carbonate derivatives of methyl *threo-* and *erythro*-9,10-dihydroxystearates as references. The derivative of the biologically derived 9,10-dihydroxystearate cochromatographed with the cyclic carbonate derivative of methyl *threo*-9,10-dihydroxystearate (retention time, 17.2 min; retention time of cyclic carbonate derivative of methyl *erythro*-9,10-dihydroxystearate, 18.1 min).

On the basis of these data, the epoxy acid isolated from human leukocytes was assigned the structure *cis*-9,10-epoxystearic acid. To determine the absolute configuration of biologically derived methyl 9,10-epoxystearate, a sample (≈20 μg) was converted into a mixture of methyl 9-hydroxy-10-octadecenoate and methyl 10-hydroxy-8-octadecenoate using a modification of a previously described procedure for conversion of epoxy derivatives into allylic alcohols (12). The (−)-menthoxycarbonyl derivaties of the allylic hydroxy esters were subjected to oxidative ozonolysis. The esterified product was analyzed by GLC.

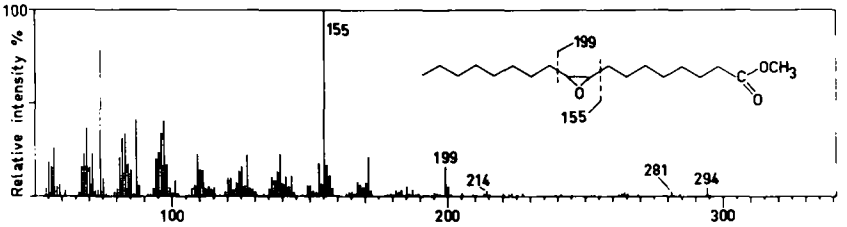

FIG. 1. Mass spectrum of methyl 9,10-epoxystearate derived from endogenous 9,10-epoxystearic acid in human leukocytes.

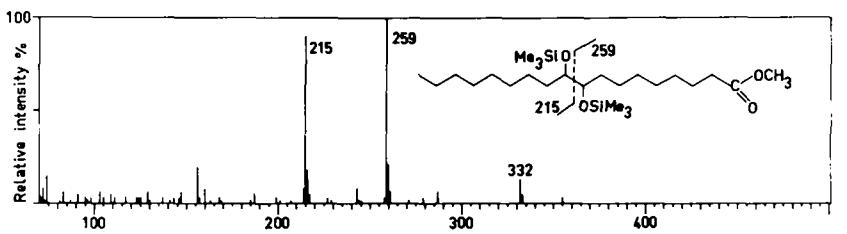

FIG. 2. Mass spectrum of the Me_3Si derivative of methyl 9,10-dihydroxystearate prepared by hydrolysis of endogenous 9,10-epoxystearic acid in human leukocytes.

This analysis showed that dimethyl 2-hydroxysebacate (derived from methyl 9-hydroxy-10-octadecenoate) and methyl 2-hydroxydecanoate (derived from methyl 10-hydroxy-8-octadecenoate) were both racemic. Accordingly, the parent methyl *cis*-9,10-epoxystearate was also racemic.

Quantitative determination of cis-*9,10-epoxystearic acid.* Methyl *cis*-9,10-epoxystearate, 0, 01, 0.2, 0.4 and 1.0 μg, was added to 2 μg of methyl [9,10,12,13-$^2H_4$]*cis*-9,10-epoxystearate. The mixtures of unlabeled and deuterated 9,10-epoxystearates were converted into methyl *threo*-9,10-dihydroxystearates by successive treatments with formic acid, sodium hydroxide and diazomethane, as described above. The Me_3Si derivatives were subjected to GC–MS. The mass region 207–225 was scanned repeatedly (ca. 10 spectra). Intensities of the ion at *m/e* 215 (protium form, $Me_3SiO^+=CH-CH_2-CH_2-CH_2-C_5H_{11}$) and of the ion at *m/e* 218 (deuterium form, $Me_3SiO^+=C^2H-CH_2-CH^2H-CH^2H-C_5H_{11}$) were summed, and the ratio of total intensities of ions 215/218 was calculated. Figure 3 shows a plot of the ratio of ion intensities vs the ratio of protium/deuterium forms in the samples analyzed. The slope was 1.06, and the y-intercept was 0.01.

For quantitative determination of *cis*-9,10-epoxystearic acid, suspensions of human leukocytes (5 ml; 10^8 cells/ml) were added to 20 vol of chloroform/methanol (2:1, v/v) containing 10 μg of deuterated methyl *cis*-9,10-epoxystearate. Extraction and alkaline hydrolysis were carried out as described above. The esterified material was subjected to TLC and the purified 9,10-epoxystearate was converted into methyl *threo*-9,10-dihydroxystearate. Analysis of the Me_3Si derivative by mass spectrometry was carried out as described above. The amount of non-deuterated dihydroxystearate, corresponding to endogenous 9,10-epoxystearate, was calculated from the ratio of intensities of ions 215/218 using the standard curve (Fig. 3). The results of eight such determinations using different batches of leukocytes gave a mean content of *cis*-9,10-epoxystearic acid in human leukocytes of 5.1 ± 2.2 μg (SD) per 10^9 cells. In another set of experiments, saponification of the leukocyte lipids was omitted. In this case, the amount of 9,10-epoxystearic acid detected was 0.4 μg (range 0.1–1.1) per 10^9 cells (n = 5). Thus, the amount of unesterified *cis*-9,10-epoxystearic acid was less than 10% of the esterified epoxy acid. In agreement with this finding, incubation of human leukocytes with *cis*-9,10-epoxystearic acid led to incorporation of the epoxy acid in leukocyte lipids. As seen in Table 1, the recovery of added *cis*-9,10-epoxystearic acid was 51% when the leukocyte lipids were hydrolyzed, as compared with 7% when hydrolysis was omitted.

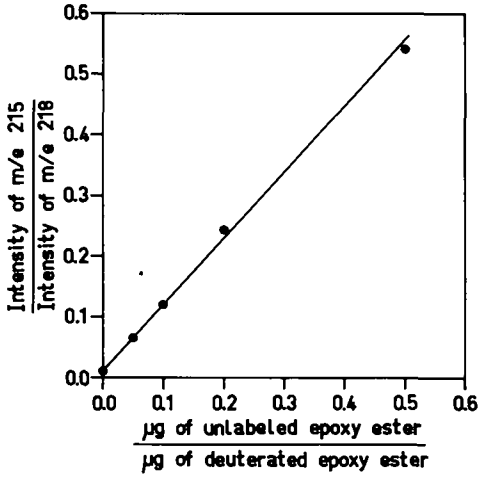

FIG. 3. Plot of the ratio of ion intensities, *m/e* 215/218 vs the ratio of protium/deuterium forms. The slope was 1.06, and the y-intercept was 0.01.

DISCUSSION

The presence of optically active 9,10-epoxystearic acid, 9,10-epoxy-12-octadecenoic acid (coronaric acid) and 12,13-epoxy-9-octadecenoic acid (vernolic acid) in certain plant oils is well documented (13). Recently, leukocytes from different animal species including man were found to produce 9,10-epoxy-12-octadecenoic acid and 12,13-epoxy-9-octadecenoic acid. The 9,10-epoxy acid was the predominant one and was named "leukotoxin" (1–2). In addition, guinea pig peritoneal leukocytes were reported to release large amounts (1.7–6.8 fmol/cell) of leukotoxin (free acid) when treated with aqueous ethanol (2).

In the present study, the occurrence of 18-carbon monoepoxy acids in lipids of human leukocytes was examined. Total leukocyte lipids were saponified, and the product was esterified by treatment with diazomethane and subjected to TLC. The TLC fraction containing monoepoxy esters was analyzed by GC–MS, either directly or following conversion of the epoxide function into the vicinal diol. The major epoxy acid was identified as 9,10-epoxystearic acid by GC–MS using the authentic compound as reference. Hydrolysis of the epoxy acid afforded *threo*-9,10-dihydroxystearic acid, thus showing that the epoxide group had the *cis* configuration. Steric analysis using a recently developed method (Fahlstadius, P. and Hamberg, M., to be published) demonstrated that the biologically derived 9,10-epoxystearate was largely racemic, i.e., a mixture of methyl 9(*S*),10(*R*)- and 9(*R*),10(*S*)-epoxystearates.

TABLE 1

Incorporation of Exogenous *cis*-9,10-Epoxystearic Acid into Leukocyte Lipids

Condition	Amount of 9,10-epoxy-stearic acid (μg)	Recovery of added 9,10-epoxy stearic acid (μg)	(%)
I	0.1	—	—
II	0.4	0.3	7
III	3.4	—	—
IV	5.3	1.9	51

Human leukocytes (5 ml, 10^8 cells/ml) were incubated either with or without 3.8 μg of *cis*-9,10-epoxystearic acid for 30 min at 37°C. The incubate was worked up and 9,10-epoxystearic acid was quantified, as described in the text. Mean values of the amounts of 9,10-epoxystearic acid in 3 experiments are shown.

I = no hydrolysis of total leukocyte lipids, no 9,10-epoxystearic acid added. II = no hydrolysis of total leukocyte lipids, 3.8 μg of 9,10-epoxystearic acid added. III = hydrolysis of total leukocyte lipids as described in the text, no 9,10-epoxystearic acid added. IV = hydrolysis of total leukocyte lipids, 3.8 μg of 9,10-epoxystearic acid added.

In addition to 9,10-epoxystearate, human leukocyte lipids were found to contain small amounts of 9,10-epoxy-12-octadecenoic acid or "leukotoxin" (1,2). The ratio of 9,10-epoxystearic acid to 9,10-epoxy-12-octadecenoic acid was 5-10:1.

A mass spectrometric method for quantitative determination of *cis*-9,10-epoxystearic acid in human leukocytes was developed. With this method, the content of ester-bound *cis*-9,10-epoxystearic acid in human leukocytes was found to be 5.1 ± 2.2 μg (SD)/10^9 cells. On the other hand, much smaller amounts of nonesterified epoxystearic acid could be detected (0.4 μg [range 0.1-1.1]/10^9 cells). This result was in agreement with the finding that *cis*-9,10-epoxystearic acid incubated with leukocyte suspensions was rapidly incorporated into leukocyte lipids. An undefined linoleic acid hydroperoxide was recently suggested to be the precursor of 9,10-epoxy-12-octadecenoic acid (2). However, it seems more likely that formation of monoepoxy acids in leukocytes occurs by a monooxygenase-catalyzed epoxidation of the appropriate unsaturated fatty acid. Conversions of oleic acid (14), linoleic and α-linolenic acids (15), as well as arachidonic acid (16,17) into monoepoxy acids in the presence of liver microsomes have been well-documented. The reaction involves cytochrome P-450 and results in the formation of a mixture of regio- and stereoisomeric (18) epoxy derivatives. The pathway in the biosynthesis of ester-bound *cis*-9,10-epoxystearate in leukocytes may consist of a monooxygenase-catalyzed epoxidation of free oleic acid followed by incorporation of the fatty acid epoxide in leukocyte lipids. Alternatively, oleate esterified in leukocyte lipids may be directly epoxidized.

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REFERENCES

1. Ozawa, T., Hayakawa, M., Takamura, T., Sugiyama, S., Suzuki, K., Iwata, M., Taki, F., and Tomita, T. (1986) *Biochem. Biophys. Res. Commun. 134,* 1072-1078.
2. Hayakawa, M., Sugiyama, S., Takamura, T., Yokoo, K., Iwata, M., Suzuki, K., Taki, F., Takahashi, S., and Ozawa, T. (1986) *Biochem. Biophys. Res. Commun. 137,* 424-430.
3. Nelson, N.S., and Blumbergs, J.H. (1964) *J. Org. Chem. 29,* 1976-1979.
4. Argoudelis, C.J., and Perkins, E.G. (1969) *Lipids 3,* 379-381.
5. Hamberg, M., Herman, R.P., and Jacobsson, U. (1986) *Biochim. Biophys. Acta 879,* 410-418.
6. Rådmark, O., Shimizu, T., Jörnvall, H., and Samuelsson, B. (1984) *J. Biol. Chem. 259,* 12339-12345.
7. Hamberg, M. (1987) *Biochim. Biophys. Acta 920,* 76-84.
8. Hamberg, M., Herman, C.A., and Herman, R.P. (1986) *Biochim. Biophys. Acta 877,* 447-457.
9. Hamberg, M. (1971) *Anal. Biochem. 43,* 515-526.
10. Ryhage, R., and Stenhagen, E. (1960) *Arkiv. Kemi 15,* 545-574.
11. Kleiman, R., and Spencer, G.F. (1973) *JAOCS 50,* 31-38.
12. Sharpless, K.B., and Lauer, R.F. (1973) *J. Am. Chem. Soc. 95,* 2697-2699.
13. Powell, R.G., Smith, C.R., and Wolff, I.A. (1967) *Lipids 2,* 172-177.
14. Watabe, T., Ueno, Y., and Imazumi, J. (1971) *Biochem. Pharmacol. 20,* 912-913.
15. Oliw, E.H. (1983) *Biochem. Biophys. Res. Commun. 111,* 644-651.
16. Oliw, E.H., Guengerich, F.P., and Oates, J.A. (1982) *J. Biol. Chem. 257,* 3771-3781.
17. Chacos, N., Falck, J.R., Wixtrom, C., and Capdevila, J. (1982) *Biochem. Biophys. Res. Commun. 104,* 916-922.
18. Falck, J.R., Manna, S., Jacobson, H.R., Estabrook, R.W., Chacos, N., and Capdevila, J. (1984) *J. Am. Chem. Soc. 106,* 3334-3336.

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Effects of Dietary Palm Oil on Arterial Thrombosis, Platelet Responses and Platelet Membrane Fluidity in Rats

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Wistar rats were fed a control diet containing 5 energy % (en %) sunflowerseed oil or diets containing 50 en % of either palm oil, rich in saturated fatty acids, or sunflowerseed oil, high in linoleic acid, for at least eight weeks. Arterial thrombosis tendency, measured by the aorta loop technique, tended to be lowered by the palm oil diet and was lowered significantly by the sunflowerseed oil diet, compared with the control. Aggregation of platelets in whole blood activated with collagen was not altered by palm oil feeding, but was enhanced in the sunflowerseed oil group, compared with the control. The concomitant formation of thromboxane A_2 was decreased by palm oil feeding, although formation of prostacyclin did not change; the ratio of thromboxane/prostacyclin formed was decreased significantly in the palm oil group. Compared with the control diet, platelet membrane fluidity, measured by fluorescence polarization, was not altered in the palm oil group and was significantly increased only by sunflowerseed-oil feeding. Thus, although palm oil contains about 50% saturated fatty acids, it did not increase arterial thrombosis tendency and tended to decrease platelet aggregation, as compared with highly polyunsaturated sunflowerseed oil.
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The type of fat in the diet has been shown to influence arterial thrombosis (1,2). Using an in vivo model for arterial thrombogenesis in rats, we have previously demonstrated that, in general, diets high in saturated fats promote arterial thrombosis, but dietary polyunsaturated fatty acids of the n-6 and n-3 families have antithrombotic effects (2,3).

Sunflowerseed oil contains large amounts of linoleic acid [18:2(n-6)], and diets enriched in this oil have a consistent antithrombotic effect, significantly increasing the obstruction time of aortic loops in rats (2). In contrast with this observation, dietary sunflowerseed oil enhances, in vitro, collagen-induced aggregation of blood platelets (4,5), cell fragments that play important roles in arterial thrombosis (6,7). This increased platelet aggregability may be due to the relatively increased production of the proaggregatory prostanoid thromboxane A_2 (TXA_2), a greater production than can be explained on the basis of the platelet phospholipid content of the thromboxane precursor, arachidonic acid (8). We have recently reported that platelets from rats fed diets high in sunflowerseed oil have an increased overall membrane fluidity (5) and, as a result, an extracellular "message," brought about by the interaction between collagen and the platelet membrane, may be transmitted more efficiently across the membrane to create a higher intracellular response (formation of TXA_2). Consequently, feedback amplification upon platelet stimulation may be enhanced.

This study describes investigations into the effects of another dietary fat, palm oil, that also has been shown to have antithrombotic properties (2). Palm oil is unusual in having an antithrombotic effect, as it contains a low ratio of polyunsaturated to saturated fatty acids; it consists of about 10% linoleic acid and 50% saturated fatty acids (mainly palmitic acid [16:0]). We have examined the effects of dietary palm oil on rat platelet aggregation and prostanoid production in response to stimulation with collagen in whole blood, and on fatty acid composition and membrane fluidity of the platelets, in an attempt to account for the antithrombotic effect of palm oil.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats (specific-pathogen-free), aged five weeks, were fed, ad libitum, one of the following three diets for at least eight weeks: a diet containing 5% of its digestible energy (en %) as sunflowerseed oil—the control diet; a diet containing 50 en % sunflowerseed oil—the sunflowerseed oil diet (which is high in polyunsaturated fatty acids); or a diet containing 50 en % palm oil—the palm oil diet (which is high in saturated and monounsaturated fatty acids). All diets contained 23 en % casein; the remaining calories were contributed by corn starch. Other dietary components (minerals, vitamins, and cellulose) were added, as previously described (2). The diets were freshly made at least once a week, and were stored at 4°C. The rats were given tap water, ad libitum.

The fatty acid compositions of the dietary fats, determined by gas liquid chromatography (GLC) after methylation (See *Lipid analyses* in this section.), are given in Table 1.

All dietary components, except the palm oil (from the Palm Oil Research Institute of Malaysia, Kuala Lumpur, Malaysia), were provided by Unilever Research, Vlaardingen, The Netherlands.

TABLE 1

Fatty Acid Composition (%) of Dietary Fats

	Dietary fat	
Fatty acid	Palm oil	Sunflowerseed oil
14:0	1	
16:0	44	6
18:0	5	4
18:1(n-9)	39	22
18:2(n-6)	10	66
18:3(n-3) + 20:0	1	1
22:0		1

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; GLC, gas liquid chromatography; OT, obstruction time.

Measurement of arterial thrombosis tendency. After a feeding period of eight to nine weeks, the arterial thrombosis tendency of the rats was measured using the aorta loop technique, as previously described (9). The time between insertion of the loop and complete obstruction of blood flow through the loop, indicated by a change in the color of the blood from red to dark blue or black, is defined as the obstruction time (OT).

Platelet aggregation in whole blood. During insertion of the aorta loop, blood (1.35 ml) was collected via the loop into a prewarmed syringe containing 3.8% trisodium citrate (0.15 ml) (5). This anticoagulated blood was transferred to an aggregometer cuvette in the thermostated (37°C) cuvette holder of a whole blood aggregometer (Model 500, Chrono-log Corp., Havertown, PA). Five min after blood collection, collagen (3.3 μg/ml, final concentration; Hormon Chemie, Munich, FRG) was added, and the change in impedance, which reflects platelet aggregation around platelets adherent to the electrodes (10,11), was recorded for 15 min.

Measurement of prostanoid formation in collagen-activated whole blood. At completion of an aggregation measurement, the blood was immediately transferred to a small tube and centrifuged at 9,600 × *g* for 2 min. The platelet-free plasma was collected and stored at −20°C until the measurement of thromboxane B_2 (TXB_2), the stable breakdown product of TXA_2, using a radioimmunoassay kit (NEK-007, New England Nuclear, Dreieich, FRG) (5), and measurement of 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin, also using a radioimmunoassay kit (NEK-008, NEN).

Platelet preparation. Suspensions of washed platelets were prepared from blood obtained by abdominal aortic puncture of rats that had been anesthetized with ether. The animals were exsanguinated at least three weeks after insertion of the aorta loop, that is, after a feeding period of at least 12 weeks. Six parts of blood were collected into one part of the anticoagulant acid-citrate-dextrose (12) and suspensions of washed platelets were prepared essentially according to the method of Ardlie and coworkers (13), as described previously (5). The final resuspension medium of the platelets was Tyrode solution containing 5 mM HEPES, pH 7.35; a portion of the final platelet suspension was used for fluidity measurements and, in some cases, the remainder was centrifuged at 1,300 × *g* for 10 min. The platelet pellet was resuspended in 0.5 ml of 139 mM NaCl, 8 mM Na_2EDTA, and the suspension was stored under N_2 at −20°C until lipid analyses were done.

Fluidity measurements. Platelet membrane fluidity was determined by the method of fluorescence polarization using the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes Inc., Junction City, OR) (5,14, 15). One volume of DPH dispersion (1 μM) was added to one volume of platelet suspension (10^8 platelets/ml) and the mixture was incubated at 37°C for 1 hr. Steady-state fluorescence-polarization measurements were done at 37°C using an Aminco-Bowman spectrofluorimeter equipped with Glan-Thompson prism polarizers in the excitation and emission beams and r_s (steady-state fluorescence anisotropy) was determined, as described previously (5). Corrections were made for scattering of polarized exciting light. To correct for slight day-to-day variations in the absolute values of r_s, results are expressed as comparisons with the control diet.

Lipid analyses. EDTA (0.5 ml of a 1% solution) was added to thawing platelet preparations (0.5 ml). A tracer amount of tritiated cholesterol ([1,2,6,7-^{3}H(N)]-cholesterol; NEN) was added to the samples to enable monitoring of recovery, and lipids were extracted according to the method of Bligh and Dyer (16). Butylated hydroxytoluene (0.005%, final concentration) was added to all organic solvents used in the lipid analyses. The phosphorus content of 5% of the total lipid extract was quantitated in duplicate, using a modification of the Fiske/Subbarow technique (17), and total platelet phospholipid content was calculated.

Cholesterol and total phospholipids were separated from other lipids by thin layer chromatography and free cholesterol was determined as described previously, correcting for recovery of tritiated cholesterol (5).

Total platelet phospholipids were eluted and transmethylated with boron trifluoride. Methyl esters of the fatty acids from the phospholipids were extracted with pentane and were analyzed by GLC with a Hewlett-Packard 5840A gas chromatograph using columns packed with 5% DEGS on Chromosorb WHP, 100/120 mesh (Chrompack, Middelburg, The Netherlands) and 2% Apiezon L on Chromosorb WHP, 100/120 mesh, as previously described (5).

Using computer-assisted analysis, chromatograms were corrected for blank runs originating from extraction of platelet-suspending medium and were adjusted with respect to the internal standard, the methyl ester of 15:0. Results are expressed as area percentage of total fatty acids. Identifications were made by comparison of retention times with those of standard mixtures of fatty acid methyl esters (from Unilever Research and Chrompack).

Analysis of data. Because OTs of aorta loops show a log-normal pattern of distribution (2), logarithmic transformation of these values was necessary for statistical analysis. The same was found to be true for TXB_2 and 6-keto-$PGF_{1\alpha}$ formed in whole blood in response to collagen. Analysis of variance, Bonferroni inequality test, Student's t-test or paired t-test were used as indicated in the Results and Discussion section to determine significance of differences. Two-tailed tests were performed.

RESULTS AND DISCUSSION

At the time of insertion of the aorta loops, that is, after a feeding period of eight to nine weeks, there was no difference in mean body weights (approximately 370 g) of the rats fed the three different diets.

Increasing the amount of sunflowerseed oil in the diet from 5 en % to 50 en % was associated with a significant prolongation of the mean log OT of the aorta loops ($P < 0.05$) (Fig. 1), indicating a decreased arterial thrombosis tendency. Rats fed the palm oil diet tended to have longer mean log OTs also, but this difference did not reach statistical significance ($P = 0.10$). Thus, palm oil feeding again tended to lower arterial thrombosis tendency. That a significant lowering of arterial thrombosis tendency was not observed in the present study may have been due to factors other than diet. For example, the variability in OTs of aorta loops is quite high and depends on the skill of the surgeon who inserts the loops. In an earlier study (3), feeding of 50 en % palm oil significantly lowered arterial thrombosis tendency compared with feeding

high-fat (50 en %) diets containing two different types of saturated triglycerides, whale oil or hydrogenated coconut oil. Further confirmation of the antithrombotic effect of palm oil was obtained in a later study (18) in which palm oils again prolonged the OT, compared with a low-fat control group (5 en % sunflowerseed oil), but no significant difference was observed in comparison with the OT of animals on a 50 en % sunflowerseed-oil diet. When results from more recent, as yet unpublished, experiments are included, arterial thrombosis tendency of rats fed a high-fat palm oil diet has been compared with that of rats fed a high-fat sunflowerseed oil diet in 10 studies, and that of rats fed control diets of low-fat (5 en %) sunflowerseed oil or 5 en % sunflowerseed oil + 45 en % hydrogenated coconut oil (This contains a high proportion of saturated fatty acids.) in five studies each. The mean OT (± SEM) of the palm oil groups was 92 ± 3.8% of that of the high sunflowerseed-oil groups ($P > 0.10$), but those of the low-fat and high-fat control groups were 65 ± 3.8% and 63 ± 2.5%, respectively ($P < 0.001$). This clearly demonstrates that dietary palm oil has a distinctive antithrombotic effect when compared with a low-fat or a high-saturated-fat diet.

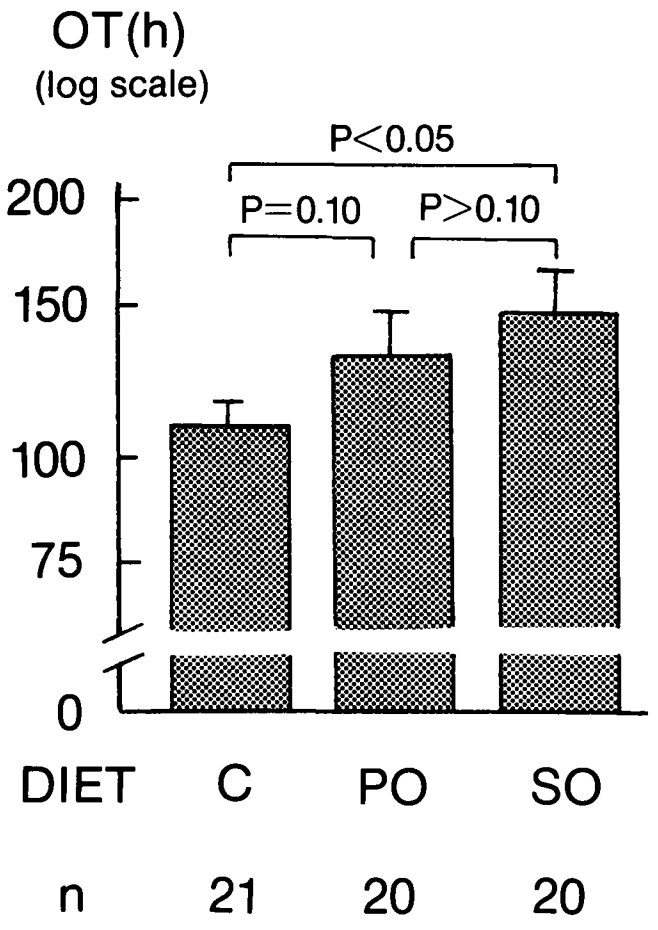

FIG. 1. Arterial thrombosis tendency, as determined by obstruction time (OT) of aorta loops of rats fed the control diet (C), the palm oil diet (PO) or the sunflowerseed oil diet (SO). See Materials and Methods for further details of the diets and aorta loop technique. Values are geometric means ± SEM. Analysis of variance and the Bonferroni inequality test (2-tailed) were used to determine significances of differences.

As measured by the maximum change in impedance, in ohms, there was a significantly greater extent of platelet aggregation in whole blood in the sunflowerseed-oil group (6.8 ± 0.81, n = 19), compared with the control group (4.25 ± 0.55, n = 19) ($P < 0.01$). There was no difference in the extent of platelet aggregation in the palm oil group (4.59 ± 0.65, n = 20), compared with the control group. The values tended to be lower than those observed for the sunflowerseed-oil group ($0.1 > P > 0.05$, means ± SEM, analysis of variance and Bonferroni inequality test).

The present study confirms earlier results (2,4,5) demonstrating that dietary sunflowerseed oil has a distinct antithrombotic effect that is associated, unexpectedly, with enhanced platelet aggregability in vitro in response to collagen. Palm oil feeding was associated with a tendency towards an antithrombotic effect, but no change in platelet aggregability, as compared with the control group. Consequently, no relationship was observed between arterial thrombosis tendency in vivo and platelet aggregability to collagen in vitro.

While sunflowerseed-oil feeding had no effect on the production of immunoreactive thromboxane or prostacyclin in collagen-activated whole blood, dietary palm oil significantly reduced thromboxane formation, leaving production of prostacyclin unaffected (Table 2). This

TABLE 2

Effect of Dietary Fat on Prostanoid Formation in Citrated Whole Blood Activated with Collagen (3.3 μg/ml)[a]

Parameter	Diet		
	Control	Palm oil	Sunflowerseed oil
TXB_2 (log ng/ml)	1.70 ± 0.066(20)	1.55 ± 0.041(20)[b]	1.64 ± 0.082(19)
(ng/ml)	50.1	35.5	43.7
6-keto-$PGF_{1\alpha}$ (log pg/ml)	2.95 ± 0.027(20)	2.99 ± 0.039(16)	3.05 ± 0.030(14)
(pg/ml)	891	977	1122
TX/PG ratio[c]	64.3 ± 7.32(20)	40.2 ± 4.34(13)[b]	61.8 ± 11.40(14)

[a] Values are means ± SEM. Number of determinations is given in parentheses.

[b] Significantly different than control ($P < 0.01$, Bonferroni inequality test).

[c] Calculated on the basis of nontransformed data.

resulted in a significantly lower thromboxane/prostacyclin ratio (Table 2), which has been suggested to determine platelet reactivity and thrombosis tendency (19). It is unlikely that the reduced thromboxane formation by collagen-stimulated platelets from rats fed the palm oil diet was due to a lower platelet count in these animals, because similar numbers of platelets were recovered from the rats fed the different diets (data not shown).

The formation of 6-keto-$PGF_{1\alpha}$ in collagen-activated whole blood most likely reflects prostacyclin production by monocytes (20). Whether these cells are activated themselves and use endogenous arachidonic acid for prostanoid formation or whether they "steal" arachidonic acid or endoperoxides from activated platelets (21) remains to be determined. Activated platelets were most likely the main source of thromboxane produced by collagen-activated whole blood.

Although thromboxane production in vitro has been demonstrated to be positively related to the arachidonic acid content of platelet phospholipids (8), the lower thromboxane production in activated whole blood of palm oil-fed animals cannot be explained on the basis of this relationship, because the arachidonic acid content of platelet phospholipids was not detectably affected by dietary palm oil (Table 3).

Lipid fluidity has been defined as the reciprocal of the lipid structural order parameter, and lower values of r_s (steady-state fluorescence anisotropy), measured using fluorescence polarization, indicate decreased structural order parameters and increased membrane fluidity (22). As observed earlier (5), the administration of a diet rich in sunflowerseed oil significantly enhanced the overall fluidity of platelet membranes, measured with the hydrophobic probe DPH (Table 4). It has been suggested that this greater fluidity promotes stimulus-response coupling, and, as a result, platelet aggregation is increased and TXA_2 production in relation to arachidonic acid availability is enhanced (5). If this is true for palm oil also, then the lower TXA_2 formation by collagen-activated platelets observed after palm oil feeding should be associated with a reduced fluidity of platelet membranes. Compared with the control, however, palm oil feeding did not modify the overall fluidity of platelet membranes (Table 4).

Parameters that are known to modulate membrane fluidity, the cholesterol/phospholipid molar ratio (C/P ratio) and the fatty acid composition of the membrane phospholipids (23,24), were determined in platelets from rats fed the control, palm-oil and sunflowerseed-oil diets. There were no significant differences among the C/P ratios of platelets from rats fed the control diet (0.60 ± 0.01, n = 3), the sunflowerseed-oil diet (0.54 ± 0.02, n = 4) or the palm-oil diet (0.56 ± 0.01, n = 5) (means ± SEM, analysis of variance). We have previously shown that the C/P ratio of platelets from rats fed the control diet and sunflowerseed-oil diet are essentially identical (5).

Feeding of the highly polyunsaturated sunflowerseed-oil diet, compared with the control diet, was associated with differences in the fatty acid composition of the total platelet phospholipids (Table 3) that reflected the fatty acid composition of the sunflowerseed oil (Table 1). For example, as shown previously (5), significantly higher levels of 18:2(n-6) (linoleic acid) and of its elongation product 24:2(n-6) were found in the sunflowerseed-oil group

TABLE 3

Fatty Acid Composition (%) of Phospholipids of Platelets from Rats Fed the Control, Palm Oil and Sunflowerseed Oil Diets[a]

Fatty acid[b]	Diet		
	Control (n = 3)	Palm oil (n = 4)	Sunflowerseed oil (n = 5)
14:0	0.6 ± 0.04	0.4 ± 0.04	0.5 ± 0.05
16:0	27.3 ± 0.15	27.5 ± 0.63	24.6 ± 0.37[e]
16:1(n-7)	1.3 ± 0.02	0.7 ± 0.08	0.9 ± 0.31
18:0	13.2 ± 1.56	12.6 ± 0.98	16.3 ± 1.12
18:1(n-9)	7.9 ± 0.65	6.7 ± 0.15	4.3 ± 0.19[e]
18:2(n-6)	4.5 ± 0.18	4.0 ± 0.36	9.9 ± 0.32[e]
20:0	NS	0.9 ± 0.13	0.7 ± 0.04
20:1(n-9)	0.5 ± 0.08	0.8 ± 0.04	0.5 ± 0.03
20:3(n-9)	0.3 ± 0.04	NS[c]	0.1 ± 0.04[e]
20:3(n-6)	0.6 ± 0.01	0.5 ± 0.07	0.7 ± 0.03[f]
20:4(n-6)	25.3 ± 0.89	25.5 ± 0.57	22.7 ± 1.34
20:5(n-3)	NS	—	0.3 ± 0.05[e]
22:0	0.6 ± 0.02	0.7 ± 0.02[e]	0.7 ± 0.03[d]
22:1(n-9)	1.0 ± 0.03	1.1 ± 0.01[f]	0.8 ± 0.04[e]
22:4(n-6)	5.3 ± 0.24	6.0 ± 1.05	5.4 ± 0.50
22:6(n-3)	0.2 ± 0.02	NS	—[d]
24:0	0.8 ± 0.03	0.7 ± 0.09	0.9 ± 0.04
24:1(n-9)	1.8 ± 0.15	2.3 ± 0.20	1.2 ± 0.03[e]
24:2(n-6)	1.0 ± 0.09	0.6 ± 0.12[d]	2.4 ± 0.16[f]
UI[g]	150.2 ± 3.5	148.3 ± 3.8	148.6 ± 4.3

[a] Values are means ± SEM. A line (—) indicates that the fatty acid was not detected, and NS indicates that the mean value was not significantly different than zero.

[b] 18:3(n-3) was not detected in the samples.

Significance of difference compared with control (Bonferroni inequality test):

[c] $P < 0.05$;

[d] $P < 0.025$;

[e] $P < 0.005$;

[f] $P < 0.001$.

[g] UI, the unsaturation index, is calculated by summing the percentage of each fatty acid in a sample multipled by the number of double bonds contained in that fatty acid (24).

(Table 3). However, feeding of the highly saturated/monounsaturated palm-oil diet was not associated with any major differences in fatty acid composition of total platelet phospholipids, compared with the control diet (Table 3).

There were no significant differences among the unsaturation indices calculated from the fatty acid compositions of total phospholipids of platelets from rats fed the three different diets (Table 3). Membrane unsaturation was also calculated in several other ways, by determining the total proportion of unsaturated fatty acids, the ratio of the unsaturation index to the proportion of unsaturated fatty acids, and the ratio of the proportion of unsaturated fatty acids to saturated fatty acids (data not shown) (24–26). There were no significant differences among the unsaturation of the membranes of the platelets from rats fed the different diets calculated in these ways. We agree with the conclusion of Lands (27), that it is difficult to correlate membrane fluidity with unsaturation of the phospholipids of membranes, calculated by means of simple formulae.

TABLE 4

Effect of Dietary Fat on Steady-State Fluorescence Anisotropy, r_s, of DPH-labeled Rat Platelets[a]

	Diet		
	Control	Palm oil	Sunflowerseed oil
Mean r_s	0.218 ± 0.0035	0.218 ± 0.0039	0.212 ± 0.0033
Paired difference than control	—	0.001 ± 0.0018	0.007 ± 0.0018[b]
Paired difference than palm oil	0.001 ± 0.0018	—	0.006 ± 0.0015[b]

[a]Means ± SEM are of 9-10 values. Paired differences are mean paired differences ± SEM of 9-10 values.
[b]Significantly different than zero ($P < 0.01$; paired t-test).

Thus, on the basis of the C/P ratios and the fatty acid data, it is not surprising that differences were not observed in membrane fluidity between the control and palm oil diets. It must be noted, however, that the membrane fluidity measured in these studies is an average membrane fluidity, as DPH partitions equally well into solid and fluid lipid domains of membranes (28), and that the membrane lipid composition that was determined is an overall composition. No information can be obtained from our studies about fluidity or lipid composition of domains of lipids that exist in the membranes (29). It remains possible that compositional and fluidity shifts occur in specific lipid domains and that such shifts were not detected with the methodology used in these studies.

This study demonstrates that palm oil, although it contains a high content of saturated fatty acids, does not promote arterial thrombus formation but, rather, tends to inhibit it. This beneficial antithrombotic effect is associated with a reduced formation of TXA_2 by activated blood platelets, although prostacyclin formation in whole blood is not affected. Collagen-induced platelet aggregation in citrated whole blood and platelet membrane fluidity are not altered in palm oil-fed animals. Upon sunflowerseed-oil feeding, however, collagen-induced platelet aggregation is enhanced and this is associated with an increased fluidity of platelet membranes. The unexpected antithrombotic effect of dietary palm oil, as well as its effect on inhibiting platelet thromboxane formation, requires further investigation.

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REFERENCES

1. Renaud, S. (1977) *Bibl. Nutr. Dieta 25,* 92-101.
2. Hornstra, G. (1982) *Dietary Fats, Prostanoids and Arterial Thrombosis,* pp. 44-62, Martinus Nijhoff, The Hague, The Netherlands.
3. Hornstra, G., and Lussenburg, R.N. (1975) *Atherosclerosis 22,* 499-516.
4. Hornstra, G. (1982) *Dietary Fats, Prostanoids and Arterial Thrombosis,* pp. 138-166, Martinus Nijhoff, The Hague, The Netherlands.
5. Rand, M.L., Hennissen, A.A.H.M., and Hornstra, G. (1986) *Atherosclerosis 62,* 267-276.
6. Chandler, A.B. (1982) in *Dietary Fats, Prostanoids and Arterial Thrombosis* (Hornstra, G., ed.), pp. 1-14, Martinus Nijhoff, The Hague, The Netherlands.
7. Packham, M.A., and Mustard, J.F. (1986) *Semin. Hematol. 23,* 8-26.
8. Hornstra, G. (1985) *Adv. Inflamm. Res. 10,* 1-6.
9. Hornstra, G., and Vendelmans-Starrenburg, A. (1973) *Atherosclerosis 17,* 369-382.
10. Cardinal, D.C., and Flower, R.J. (1980) *J. Pharmacol. Methods 3,* 135-158.
11. Ingerman-Wojenski, C.M., and Silver, M.J. (1984) *Thromb. Haemostas. 51,* 154-156.
12. Aster, R.H., and Jandl, J.H. (1964) *J. Clin. Invest. 43,* 843-855.
13. Ardlie, N.G., Packham, M.A., and Mustard, J.F. (1970) *Br. J. Haematol. 19,* 7-17.
14. Shinitzky, M., and Barenholz, Y. (1978) *Biochim. Biophys. Acta 515,* 367-394.
15. Shattil, S.J., and Cooper, R.A. (1976) *Biochemistry 15,* 4832-4837.
16. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
17. Böttcher, C.J.F., van Gent, C.M., and Pries, C.A. (1961) *Anal. Chim. Acta 24,* 203-204.
18. Hornstra, G. (1987) *Agents Actions 22,* 377-378.
19. Moncada, S., and Vane, J.R. (1978) *Br. Med. Bull. 34,* 129-135.
20. McGuire, J.C., and Sun, F.F. (1980) *Adv. Prost. Thrombox. Res. 8,* 1665-1667.
21. Defreyn, G., Deckmyn, H., and Vermylen, J. (1982) *Thromb. Res. 26,* 389-400.
22. van Blitterswijk, W.J., van Hoeven, R.P., and van der Meer, B.W. (1981) *Biochim. Biophys. Acta 644,* 323-332.
23. Shinitzky, M. (1984) in *Biomembranes* (Kates, M., and Manson, L.A., eds.) Vol. 12, pp. 585-601, Plenum, New York, NY.
24. Stubbs, C.D., and Smith, A.D. (1984) *Biochim. Biophys. Acta 779,* 89-137.
25. Berlin, E., Matusik, Jr., E.J., and Young, C. Jr. (1980) *Lipids 15,* 604-608.
26. Berlin, E., Shapiro, S.G., and Friedland, M. (1984) *Atherosclerosis 51,* 223-239.
27. Lands, W.E.M. (1980) *Biochem. Soc. Trans. 8,* 25-27.
28. Lentz, R.B., Barenholz, Y., and Thompson, T.E. (1976) *Biochemistry 15,* 4521-4528.
29. Karnovsky, M.J. (1979) *Am. J. Path. 97,* 212-221.

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Formation of Red Pigment by a Two-Step 2-Thiobarbituric Acid Reaction of Alka-2,4-dienals. Potential Products of Lipid Oxidation

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Reaction of 2,4-hexadienal, 2,4-nonadienal and 2,4-decadienal with 2-thiobarbituric acid (TBA) in aqueous acetic acid produced a 532-nm absorbing red pigment. While the 1:1 reaction of the aldehyde and TBA produced little pigment, reaction of the aldehyde with an excess amount of TBA produced significant amounts. Instant heating of the reaction mixture did not produce the pigment. However, initial reaction at 5°C and subsequent heating to 100°C produced the pigment efficiently (two-step reaction). Pigment formation required water and dissolved oxygen. The yield of the pigment from the alka-2,4-dienals was 1/10–1/20 of that from malonaldehyde. In the first step of the reaction at 5°C, the 1:1 adducts of the aldehydes at the *5*-position of TBA and several other unidentified adducts were formed. In the second step, these adducts were converted at 100°C, in the presence of water and oxygen, into the red pigment. The structure of the red pigment from 2,4-hexadienal was elucidated to be the 1:2 adduct of malonaldehyde and TBA. 2-Hexenal and *t*-butylhydroperoxide showed marked synergistic effects on the pigment formation from the alka-2,4-dienals. Red pigment formation due to the alka-2,4-dienals may be enhanced by the presence of other aldehydes and hydroperoxides.

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The 2-thiobarbituric acid (TBA) test has been widely used to measure lipid oxidation (1). Red pigment with an absorption maximum at 532 nm formed in the TBA test of oxidized lipids has been regarded as the 1:2 adduct of malonaldehyde and TBA (2–4).

Recent studies have shown that the TBA test is not specific to malonaldehyde. Malonaldehyde content in oxidized lipids determined by alternative methods are usually lower than those determined by the TBA test (5–11). It is known that monofunctional aldehydes that can be formed in oxidized lipids produce a similar red pigment (12–18). A large part of the red pigment produced by the TBA test of oxidized lipids may be due to these monofunctional aldehydes. However, the contribution of these monofunctional aldehydes to the red pigment formation has not yet been established.

In our investigations of the TBA reaction of monofunctional aldehydes, it was found that alkanals (17) and alk-2-enals (18) produce the red pigment, depending on the reaction conditions. The pigment formation from alkanals is restricted to the reactant ratio of aldehyde: TBA = 1:1 (17). Alk-2-enals produce the pigment at the reactant ratio of 1:1 in maximal yields, but significant amounts of the pigment are produced with an excess amount of TBA (18). The yield of the pigment from alk-2-enals is 1/100–1/500 of that from malonaldehyde with an excess amount of TBA.

We have now investigated the red pigment formation in the reaction of alka-2,4-dienals with TBA in aqueous acetic acid under various conditions. We found that the reaction conditions for alka-2,4-dienals to produce the pigment were greatly different from those for alkanals and alk-2-enals, and that alka-2,4-dienals produced much greater amounts of the pigment by reaction with an excess amount of TBA under the two-step reaction conditions.

MATERIALS AND METHODS

Materials. TBA and 1-butanal were obtained from Wako Pure Chemical Industries (Osaka, Japan). Malonaldehyde bis(dimethylacetal) and 2-hexenal were from the Tokyo Kasei Kogyo Co. (Tokyo, Japan). 2,4-Hexadienal, 2,4-nonadienal and 2,4-decadienal were from Aldrich Chemical Co. (Milwaukee, WI). *t*-Butylhydroperoxide (70% in water) was from Nakarai Chemicals (Kyoto, Japan). The red pigment between malonaldehyde and TBA was prepared according to the method previously reported (2,4).

Analyses. Absorption spectra were measured on a Shimadzu UV-240 or a Hitachi U-2000 spectrophotometer. Fluorescence spectra were measured with a Hitachi 650-60 fluorescence spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-400 NMR spectrometer. Signals in ^{13}C NMR spectra were assigned by distortionless enhancement by polarization transfer (DEPT) experiments. Analytical high performance liquid chromatography (HPLC) was carried out with a Shimadzu LC-5A or LC-6A liquid chromatograph equipped with a stainless steel column (4.6 mm × 25 cm) of YMC A-303 ODS (Yamamura Chemical Laboratories Co., Kyoto, Japan). The chromatograph was operated by elution with acetonitrile/0.025 M phosphate buffer (pH 3.0) (2:8, v/v) or methanol/0.04 M acetate buffer (pH 5.5) (4:6, v/v) at a flow rate of 0.8 ml/min. The peaks were detected with Shimadzu SPD-1, SPD-2A or SPD-6A spectrophotometers. Preparative HPLC of the red pigment was carried out with a Hitachi 655-11 liquid chromatograph equipped with a stainless steel column (20 mm × 25 cm) of YMC S-343 ODS, and the chromatograph was operated by elution with methanol/0.04 M acetate buffer (pH 5.5) (4:6, v/v) at a flow rate of 5.0 ml/min.

Reaction of the aldehydes with an excess amount of TBA in aqueous acetic acid. The TBA solution (0.4%) in aqueous acetic acid (4.9 ml) was placed in a screw cap tube (13 ml-content). For the two-step reaction, the tube was precooled to 5°C and 0.1 ml of the aldehyde solution in acetic acid was added. The mixture was kept at 5°C for 1 hr and then heated at 100°C for 20 min. For the one-step reaction, the tube containing the TBA solution was preheated at 100°C, followed by addition of the aldehyde solution. The mixture was then heated at 100°C for 20 min.

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Abbreviations: TBA, 2-thiobarbituric acid; DEPT, distortionless enhancement by polarization transfer; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance.

Isolation of 1:1 adducts X between the alka-2,4-dienals and TBA. Seven mmol of 2,4-hexadienal, 2,4-nonadienal or 2,4-decadienal was added to a solution of 0.5 g (3.47 mmol) of TBA in 35 ml of 50% acetic acid. A yellow precipitate that appeared during 30–60 min was collected by filtration. The precipitate from 2,4-hexadienal was dissolved in 50 ml of hot glacial acetic acid and filtered. An equal amount of water was added to the filtrate and the mixture was cooled. The precipitate was collected and washed with water to produce X_{HD} (261 mg, yield 33.2%). The precipitates from 2,4-nonadienal and 2,4-decadienal were recrystallized from ethanol to yield pure samples, X_{ND} (351 mg, yield 32.7%) and X_{DD} (295 mg, yield 30.0%), respectively.

Isolation of the red pigment formed in the reaction of 2,4-hexadienal and TBA. A 1.5 liter of 2% acetic acid containing 0.4% TBA and 2 mM 2,4-hexadienal was kept at 5°C for 1 hr and then heated at 100°C for 20 min. The mixture was extracted twice with 50 ml of 1-butanol. The extract was evaporated to dryness and the residue was dissolved in 15 ml of dimethylsulfoxide. The resulting solution was subjected to preparative HPLC. The 532-nm-absorbing peak fractions that appeared at a retention time of 24 min were combined and evaporated to a small volume. A red-violet precipitate was collected by centrifugation at 3,000 rpm for 1 min and washed well with water. The precipitate was dried at 50°C over P_2O_5 in vacuo and yielded 12.2 mg.

RESULTS

Reaction of alka-2,4-dienals with an equivalent amount of TBA. When 0.5 mM 2,4-hexadienal, 2,4-nonadienal or 2,4-decadienal was allowed to react with an equivalent amount of TBA in 15% acetic acid at 100°C for 0.25–6 hr, the reaction mixture turned slightly orange, exhibiting absorption maxima at 495 nm. The spectra from reaction mixtures of alkanals (17) and alk-2-enals (18) heated under the same conditions exhibited absorption maxima at 532 nm. The amount of red pigment formed from alka-2,4-dienals, estimated by absorbance at 532 nm, was lower than that from alkanals (17) and alk-2-enals (18).

Two-step reaction of alka-2,4-dienals with an excess amount of TBA. Treatment of alka-2,4-dienals with an excess amount of TBA in aqueous acetic acid at 100°C produced a red pigment with an absorbance at 532 nm, but the results were not reproducible. After studying the reaction conditions, it was found that allowing the reaction mixture to stand at a relatively low temperature prior to heating (two-step reaction) effectively produced the pigment.

Figure 1A shows absorption spectra of the reaction mixtures of 0.1 mM 2,4-hexadienal and 0.4% (28 mM) TBA in 2% acetic acid. Although instant heating of the reaction mixture at 100°C did not exhibit any significant absorbance at 532 nm, treatment at 5°C prior to heating gave markedly greater absorbance. The reaction of 2,4-nonadienal and 2,4-decadienal under these conditions also yielded a pigment with the same absorption spectrum. Absorption spectra of the reaction mixtures of the alka-2,4-dienals were identical with that of the reaction mixture of malonaldehyde, which was not pretreated (Fig. 1B). Absorption spectra of the reaction mixture of 2-hexenal, showing absorption maxima at 455, 495 and

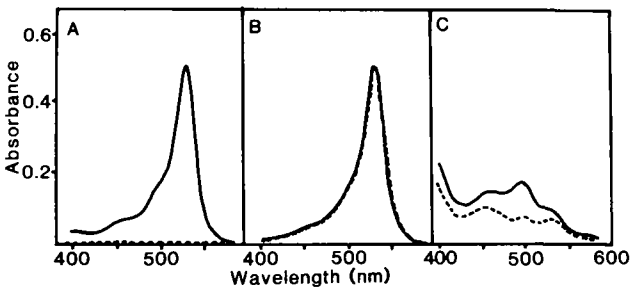


FIG. 1. Absorption spectra of the reaction mixtures of 0.1 mM 2,4-hexadienal (A), 0.0038 mM malonaldehyde bis(dimethylacetal) (B), and 2 mM 2-hexenal (C) with 0.4% (28 mM) TBA in 2% acetic acid. The reaction mixtures were instantly heated at 100°C for 20 min (– – – –), or treated at 5°C for 1 hr and subsequently heated at 100°C for 20 min (——).

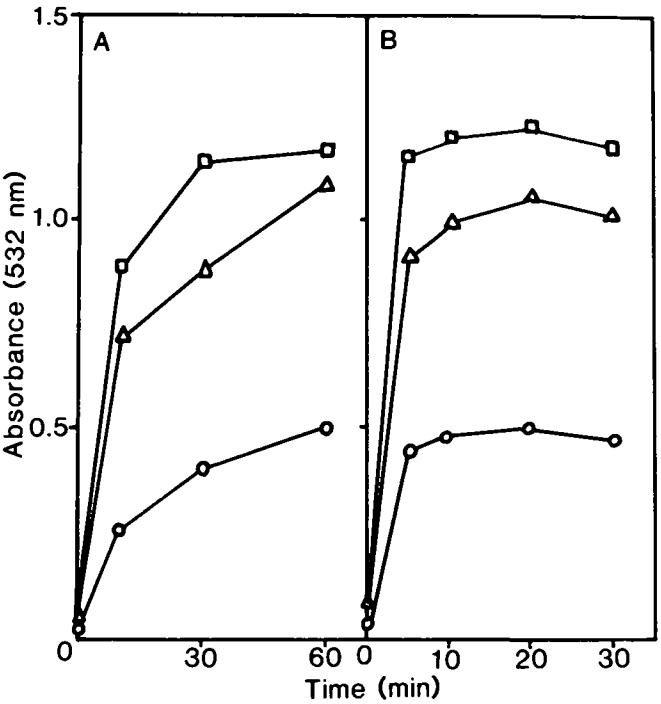


FIG. 2. Time courses of absorbance at 532 nm of the reaction mixtures of 0.08 mM 2,4-hexadienal (○), 2,4-nonadienal (△) and 2,4-decadienal (□), with 0.4% TBA in 2% acetic acid. A: Time courses in the treatment at 5°C prior to heating at 100°C for 20 min. B: Time courses in the heating at 100°C after treatment at 5°C for 1 hr.

532 nm (18), were slightly but not excessively influenced by the pretreatment (Fig. 1C).

Figure 2 shows the time courses of the pigment formation in two-step reaction of the alka-2,4-dienals. The absorbances of the reaction mixtures heated at 100°C for 20 min increased with the pretreatment time (Fig. 2A), and those treated at 5°C for 1 hr increased with subsequent heating (Fig. 2B). Treatment of the reaction mixtures at 5°C for 1 hr and subsequent heating at 100°C for 20 min produced the pigment effectively. When the temperature of the pretreatment was raised from 5°C to 25 or 37°C, formation of the pigment was retarded but still greater than that without the pretreatment. When

the temperature was lowered to 0°C, reproducible absorbance could not be obtained because of precipitation of the TBA into the reaction mixtures.

The pigment formation in the two-step TBA reaction of the alka-2,4-dienals depended on the water content of the reaction mixture. For instance, absorbance of the reaction mixture of 0.08 mM 2,4-hexadienal at 532 nm in 2, 20, 40 and 60% acetic acid was 0.50, 0.15, 0.07 and 0.05, respectively. Formation of the pigment increased progressively with the water content. The pigment formation required dissolved oxygen in the reaction mixtures. Thus, the absorbance did not increase when oxygen in the reaction mixtures was removed by bubbling nitrogen gas prior to the reaction. Water and oxygen were also necessary for the pigment formation from alkanals (17) and alk-2-enals (18). Certain hydrolytic and oxidative mechanisms may be involved in the red pigment formation from these monofunctional aldehydes.

Calibration curves of the absorbance of the two-step TBA reaction of the alka-2,4-dienals (Fig. 3) were not linear but concave. Efficacy for the pigment formation was greater as the carbon number of the aldehydes increased. The absorbance of the two-step TBA reaction mixtures of malonaldehyde and 2-hexenal linearly increased with their concentrations. The concentrations of the aldehydes that gave absorbance 1.0 was 0.1–0.2 mM for the alka-2,4-dienals, 0.007 mM for malonaldehyde and 20 mM for 2-hexenal. Thus, the potency of the pigment formation was in the order of malonaldehyde > alka-2,4-dienals > alk-2-enals under the two-step reaction conditions.

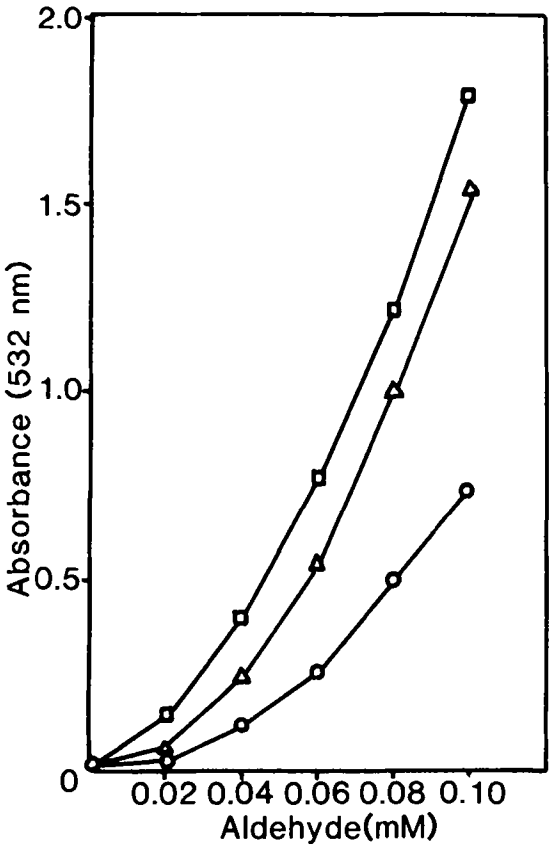


FIG. 3. Calibration curves of absorbance at 532 nm of the reaction mixtures of 2,4-hexadienal (○), 2,4-nonadienal (△) and 2,4-decadienal (□), with 0.4% TBA in 2% acetic acid. The reaction mixtures were treated at 5°C for 1 hr and subsequently heated at 100°C for 20 min.

Presence of intermediary adducts for the red pigment in the first step reaction of alka-2,4-dienals. When a 200 mM mixture of alka-2,4-dienal and 100 mM TBA in 50% acetic acid was allowed to stand at room tempeature, adduct X precipitated. Adduct X_{HD} from 2,4-hexadienal, X_{ND} from 2,4-nonadienal and X_{DD} from 2,4-decadienal were isolated, and their structures were elucidated to be the 1:1 adducts of the aldehyde at the *5*-position of TBA. Physico-chemical properties of these adducts are listed in Table 1. The mass spectra of these adducts revealed the molecular ion peaks corresponding to the indicated formulas. In the ^1H NMR spectrum of each of these adducts, one proton at ca. 8.1 ppm, one proton at ca. 7.9 ppm, one proton at ca. 7.2 ppm and two protons at ca. 6.5 ppm were assignable to the conjugated triene system *(a–e)*. Protons appearing in the higher fields indicated the presence of alkyl residue R. In the ^{13}C NMR spectrum of X_{DD}, signals at 179.6, 161.9 and 161.4 ppm were assignable to the carbons at the *2, 4* and *6*-positions of the TBA ring, five signals between 128 and 156 ppm were to the conjugated triene system *(a–e)*, a signal at 115.8 ppm was to the carbon at the *5*-position of the TBA ring, four signals between 23.3 and 34.2 ppm were to the alkyl chain *(f–i)*, and a singal at 14.3 ppm was to the terminal methyl group *(j)*.

Absorption spectra of these adducts in chloroform revealed maxima at ca. 410 nm. The adducts were unstable in methanol and aqueous acetic acid. For instance, the absorption maximum of X_{HD} in methanol shifted from 385 to 348 nm with an isosbestic point at 359 nm at room temperature for 70 min. The absorbance at the maximum wavelength (409 nm) of X_{HD} in 50% acetic acid was lowered to a half value at room temperature for 4 hr.

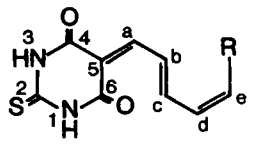
Intermediates for the red pigment produced in the first step of the reaction of 2,4-hexadienal were analyzed by HPLC. Thus, a mixture of 0.5 mM 2,4-hexadienal and 2 mM TBA in 50% acetic acid was kept at 5°C and subjected to HPLC (Fig. 4A). The peak corresponding to TBA decreased and the new peaks corresponding to unidentified adducts S, T, U and V appeared after 2 hr. No significant peak corresponding to X_{HD} was observed. A mixture of 0.5 mM X_{HD} and 2 mM TBA was kept at 5°C and subjected to HPLC (Fig. 4B). The peaks due to TBA and X_{HD} decreased and the peaks due to S, T, U and V appeared after 2 hr. Unstable adduct X_{HD} may be initially produced which, in turn, reacted with TBA to form other adducts S, T, U and V. These adducts may be converted into the red pigment in the second step reaction at 100°C.

Involvement of adduct X_{HD} as an intermediate for the red pigment was demonstrated. When a mixture of 0.1 mM X_{HD} and 0.4% TBA in 2% acetic acid was heated at 100°C for 20 min, the red pigment with an absorbance 0.34 was formed. Control two-step TBA reaction of 0.1 mM 2,4-hexadienal yielded the pigment with an absorbance 0.65 (Fig. 3).

Isolation and identification of the red pigment formed in the two-step TBA reaction of 2,4-hexadienal. The reaction mixture of the two-step TBA reaction of 2,4-

TABLE 1

Physico-chemical Data of Adducts X



X

Adduct	Structure R	Formula[a]	UV max nm (ε) $CHCl_3$	Mass spectrum *m/z* (rel intensity)	^1H NMR spectrum ppm ($CDCl_3$)	^{13}C NMR spectrum ppm (d_8-tetrahydrofuran)
X_{HD}[b]	$\overset{f}{CH_3}$	$C_{10}H_{10}N_2O_2S \cdot 0.25\ H_2O$	412 (48800)	222 (100)[M^+]	1.98 (3 H, d, J = 6 Hz) *f* 6.46 (2 H, m) *d,e* 7.15 (1 H, dd, J = 10, 15 Hz) *c* 7.84 (1 H, dd, J = 15, 12 Hz) *b* 8.08 (1 H, d, J = 12 Hz) *a* 8.80 (1 H, s) } *1,3* 8.87 (1 H, s) }	
X_{ND}[c]	$\overset{f}{CH_2}\overset{g}{CH_2}\overset{h}{CH_2}\overset{i}{CH_3}$	$C_{13}H_{16}N_2O_2S \cdot 0.25\ H_2O$	412 (51900)	264 (100)[M^+] 235 (12)[$M^+ - 29$] 208 (34)	0.98 (3 H, t, J = 7 Hz) *i* 1.36 (2 H, m) *h* 1.46 (2 H, m) *g* 2.29 (2 H, m) *f* 6.45 (2 H, m) *d,e* 7.16 (1 H, dd, J = 10, 15 Hz) *c* 7.86 (1 H, dd, J = 15, 12 Hz) *b* 8.09 (1 H, d, J = 12 Hz) *a* 8.86 (1 H, s) } *1,3* 8.95 (1 H, s) }	
X_{DD}[d]	$\overset{f}{CH_2}\overset{g}{CH_2}\overset{h}{CH_2}\overset{i}{CH_2}\overset{j}{CH_3}$	$C_{14}H_{18}N_2O_2S \cdot 0.25\ H_2O$	408 (52800)	278 (100)[M^+] 249 (6)[$M^+ - 29$] 222 (84) 221 (92)][$M^+ - 59$]	0.90 (3 H, t, J = 7 Hz) *j* 1.32 (4 H, m) *h,i* 1.48 (2 H, m) *g* 2.28 (2 H, m) *f* 6.45 (2 H, m) *d,e* 7.17 (1 H, dd, J = 10, 15 Hz) *c* 7.86 (1 H, dd, J = 15 ,12 Hz) *b* 8.10 (1 H, d, J = 12 Hz) *a* 8.82 (1 H, s) } *1,3* 8.90 (1 H, s) }	14.3 *j* 23.3 29.2 32.3 *f,g,h,i* 34.2 115.8 *5* 128.4 132.0 148.7 *a,b,c,d,e* 155.1 156.0 161.4 161.9 *2,4,6* 179.6

[a] Elemental analyses (C, H, N) were within ± 0.4% of the theoretical values.
[b] From 2,4-hexadienal.
[c] From 2,4-nonadienal.
[d] From 2,4-decadienal.

hexadienal was subjected to HPLC (Fig. 5). The retention time of the peak due to the red pigment was identical with that of pigment derived from malonaldehyde. The red pigment was purified and isolated by preparative HPLC. ^{1}H and ^{13}C NMR spectra (Table 2) of the pigment confirmed that the structure was the 1:2 adduct of malonaldehyde and TBA. The ^{13}C NMR spectrum showed three signals at 176.5, 162.8 and 161.4 ppm assignable to the carbons at the *2, 4 and 6*-position of the TBA ring, two signals at 157.4 and 117.5 ppm assignable to vinyl carbons (*a* and *b*) and a signal at 101.4 ppm assignable to the carbon at the *5*-position of the TBA ring. The ^{1}H NMR spectrum revealed the presence of vinyl protons (*a* and *b*) as a doublet at 7.69 ppm and a triplet at 8.55 ppm. The data coincided with that of the 1:2 adduct of malonaldehyde and TBA (3). The absorption spectrum of the pigment showed maxima at 532, 305 and 245 nm (Fig. 6A), which were similar to those of the 1:2 adduct of malonaldehyde and TBA (2–4). The fluorescence spectrum of the pigment showed an excitation maximum at 532 nm and an emission maximum at 553 nm (Fig. 6B), which were similar to those previously reported (19).

It is interesting that the reaction of 2,4-hexadienal and TBA gave the 1:2 adduct of malonaldehyde and TBA.

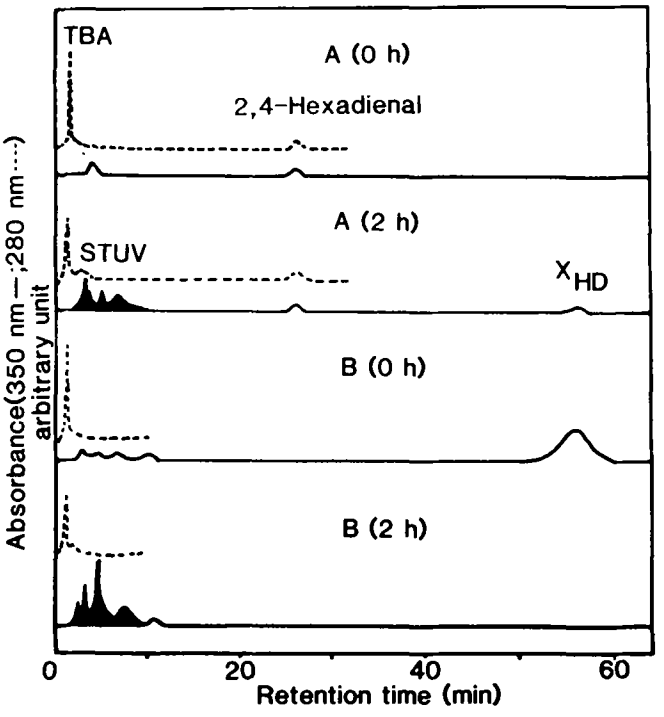


FIG. 4. HPLC patterns of the intermediates for the red pigment in the reaction of 2,4-hexadienal and TBA. A mixture of 0.5 mM 2,4-hexadienal with 2 mM TBA (A), and 0.5 mM X_{HD} with 2 mM TBA (B) in 50% acetic acid was kept at 5°C for 2 hr. The mixture was subjected to a column of YMC A-303 ODS and eluted with acetonitrile/0.025 M phosphate buffer (pH 3.0) (2:8, v/v) at flow rate of 0.8 ml/min. TBA appeared at a retention time of 1 min when detected at 280 nm, and 2,4-hexadienal and X_{HD} at a retention time of 31 min and 56 min, respectively, when detected at 350 nm. X_{HD} was kept stable under HPLC conditions.

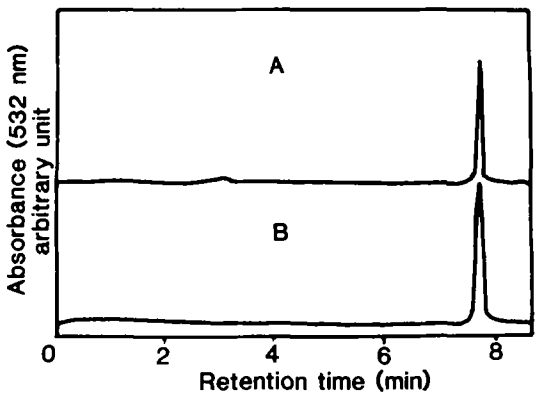
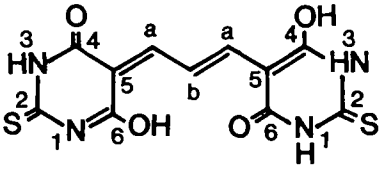


FIG. 5. HPLC patterns of the red pigment derived from 2,4-hexadienal and malonaldehyde. A mixture of 0.1 mM 2,4-hexadienal (A) and 0.05 mM malonaldehyde bis(dimethylacetal) (B) with 0.4% TBA in 2% acetic acid was kept at 5°C for 1 hr and then heated at 100°C for 20 min. The mixture was subjected to a column of YMC A-303 ODS and eluted with methanol/0.04 M acetate buffer (pH 5.5) (4:6, v/v) at a flow rate of 0.8 ml/min.

TABLE 2

^{1}H NMR and ^{13}C NMR Spectra of the Red Pigment Derived from 2,4-Hexadienal



^{1}H NMR spectrum (d_6-dimethylsulfoxide)		^{13}C NMR spectrum (d_6-dimethylsulfoxide)		
ppm	Assignment	ppm	DEPT	Assignment
7.69 (2 H, d, J = 13.7 Hz)	*a*	101.4	C	*5*
8.55 (1 H, t, J = 13.7 Hz)	*b*	117.5	CH	*b*
11.44 (bs)	*1,3,4,6*	157.4	CH	*a*
		161.4	C	
		162.8	C	*2,4,6*
		176.5	C	

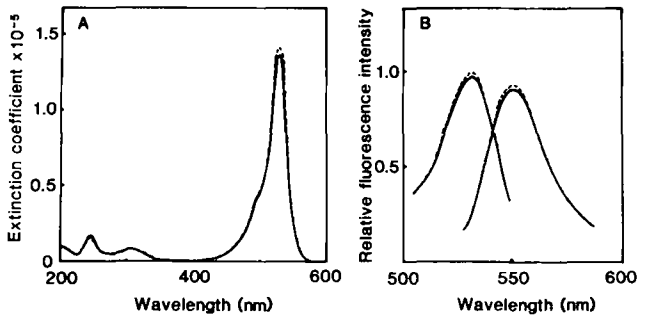
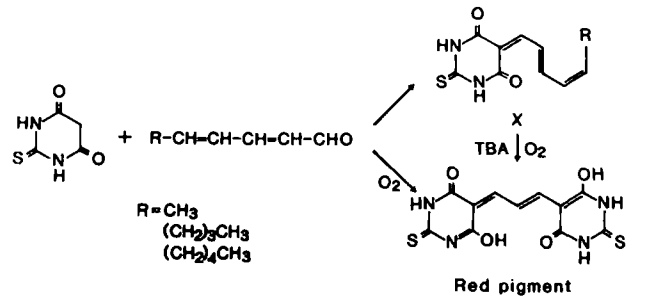


FIG. 6. Absorption spectra (A) and fluorescence spectra (B) of the red pigment derived from 2,4-hexadienal (solid line) and malonaldehyde (dotted line) in water. The red pigment from malonaldehyde was prepared according to the method previously reported (Refs. 2 and 4). Fluorescence excitation (monitored at 565 nm) and emission (monitored at 515 nm) spectra were obtained at a concentration of 0.4 μg/ml.



SCHEME 1

Hydrolysis and oxidation of the intermediary adducts may be involved in the red pigment formation in the second step of the reaction (Scheme 1).

Synergistic effects of 2-hexenal and t-butylhydroperoxide on the red pigment formation in the two-step TBA reaction of alka-2,4-dienals. The two-step TBA reaction of 2,4-hexadienal was performed in the presence of malonaldehyde, 1-butanal, 2-hexenal and *t*-butylhydroperoxide (Fig. 7). These components or functions may be involved in the oxidized lipids. Malonaldehyde (0.05 mM) which produced an absorbance of 0.80, 1-butanal (1 mM) with no absorbance, and 2-hexenal (2 mM) with an absorbance of 0.10 were added to the reaction mixtures of 2,4-hexadienal (0–0.1 mM) (Fig. 7A). The rate of increase in absorbance with the dose of 2,4-hexadienal was not influenced by malonaldehyde and little by 1-butanal. In contrast, 2-hexenal markedly potentiated the rate of increase in absorbance. Addition of 1 mM *t*-butylhydroperoxide to the reaction mixtures of 2,4-hexadienal potentiated the rate of increase in absorbance, whereas the hydroperoxide alone did not produce any red pigment (Fig. 7B). These results indicated that there were little or no interactions between 2,4-hexadienal and malonaldehyde/1-butanal but there existed dramatic synergism between 2,4-hexadienal and 2-hexenal/*t*-butylhydroperoxide.

Figure 8 shows the synergistic effects of 20 equivalent amounts of 2-hexenal on the red pigment formation in the two-step TBA reaction of three alka-2,4-dienals. In

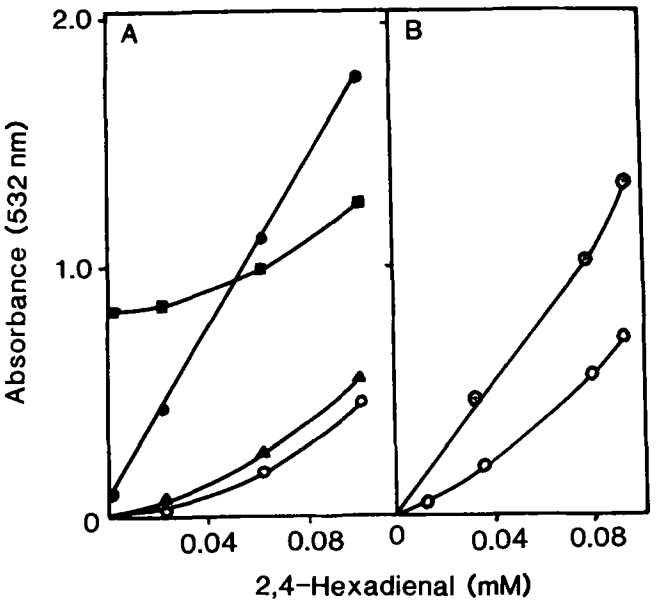


FIG. 7. Effects of malonaldehyde, 1-butanal, 2-hexenal and *t*-butylhydroperoxide on the red pigment formation in the two-step TBA reaction of 2,4-hexadienal. A: A mixture of 2,4-hexadienal with 0.4% TBA in 4% acetic acid was treated at 5°C for 1 hr and then heated at 100°C for 20 min in the presence of none (○), 0.005 mM malonaldehyde bis(dimethylacetal) (■), 2 mM 1-butanal (▲) and 2 mM 2-hexenal (●). B: A mixture of 2,4-hexadienal with 0.4% TBA in 2% acetic acid was treatecd at 5°C for 1 hr and then heated at 100°C for 20 min in the presence of none (○) and 1 mM *t*-butylhydroperoxide (⊙).

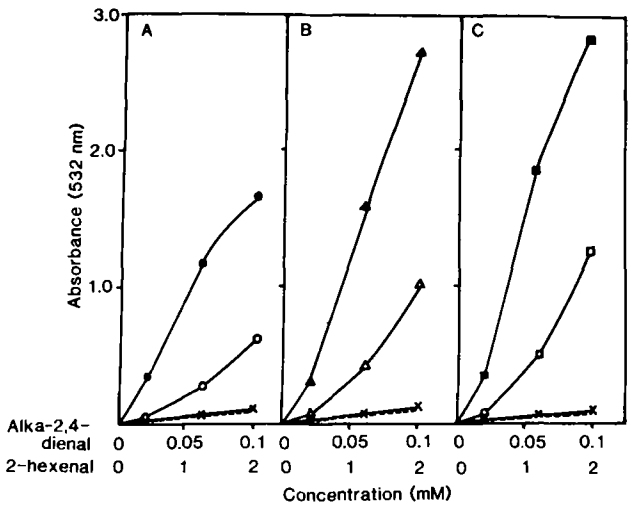


FIG. 8. Synergistic effects of 2-hexenal on the red pigment formation in the two-step TBA reaction of 2,4-hexadienal (A), 2,4-nonadienal (B) and 2,4-decadienal (C). A mixture of the alka-2,4-dienal alone (open circle, triangle and square), alka-2,4-dienal/2-hexenal (closed circle, triangle and square), and 2-hexenal alone (×), with 0.4% TBA in 2% acetic acid was kept at 5°C for 1 hr and heated at 100°C for 20 min. A mixture of alka-2,4-dienal/2-hexenal with 0.4% TBA in 2% acetic acid was instantly heated at 100°C for 20 min (– – – –).

every case, marked synergism was observed. The dose-dependent pigment formation due to the alka-2,4-dienals was increased 2-fold by addition of 20 equivalent amounts of 2-hexenal. Instant heating of the mixtures prevented

TABLE 3

Red Pigment Formation in the Reaction of Aldehydes With an Excess Amount of TBA in Aqueous Acetic Acid at 100°C

Aldehyde	Requirement for the pigment formation			Intermediates	Yield of red pigment	Synergism with	Reference
	Oxygen	Water	Pretreatment at 5°C				
Malonaldehyde	−	±	−	−	1		
Alkanals	+	+	−	W[a]	Extremely low		15
Alk-2-enals	+	+	−	X[a] → Y	1/100-1/500		16
Alka-2,4-dienals	+	+	+	X[a] → S,T,U,V	1/10-1/20	2-hexenal *t*-butylhydroperoxide	Present paper

[a]Structure established.

the pigment formation due to the alka-2,4-dienals and also abolished the synergism with 2-hexenal.

DISCUSSION

The TBA test is generally used for measurement of lipid oxidation, and the 532-nm absorbing red pigment has been widely used as an excellent index in medical and food science fields (1). The pigment is produced by the reaction of not only malonaldehyde (1-4), but also other aldehydes (12-18). Previously, we have shown that alkanals and alk-2-enals produce the red pigment, depending on the reaction conditions (17,18). It has been shown that alka-2,4-dienals produce the red pigment (13) and the yield of the pigment is very low in comparison with malonaldehyde (15). However, characteristics and contribution of the red pigment from alka-2,4-dienals have not yet been elucidated. We examined the TBA reaction of alka-2,4-dienals in aqueous acetic acid at 100°C under various conditions. It was found that there were many factors which influenced the pigment formation. The factors were the ratio of the reactants; presence of water, oxygen, other aldehydes and hydroperoxides; and time and temperature of pretreatment.

Although the pigment formation from alkanals and alk-2-enals is the highest at the aldehyde:TBA ratio 1:1 (17,18), that from alka-2,4-dienals was very low at that ratio. The pigment formation from alka-2,4-dienals was the highest in the reaction with an excess amount of TBA. The characteristics of the red pigment formation from alkanals, alk-2-enals and alka-2,4-dienals by reaction with an excess amount of TBA in aqueous acetic acid at 100°C are summarized in Table 3. Alkanals, alk-2-enals and alka-2,4-dienals all required water and dissolved oxygen for effective pigment formation, which may indicate the involvement of certain hydrolytic and oxidative mechanisms. The effective pigment formation from alka-2,4-dienals required pretreatment at 5°C, whereas that from other aldehydes did not. Thus, the two-step reaction of alka-2,4-dienals at 5°C and 100°C effectively produced the pigment. In the first step of the reaction, 1:1 adducts X may be initially formed which may be converted into other adducts such as S, T, U and V. These adducts may be converted into the red 1:2 adduct of malonaldehyde and TBA (2-4) in the second step heating (Scheme 1).

Although the calibration curves of alk-2-enals are linear (18), the calibration curves of alka-2,4-dienals were concave. Alka-2,4-dienals produced the red pigment in an amount 1/10-1/20 of that from malonaldehyde at concentrations that gave an absorbance of 1.0. This value was much higher than that from alk-2-enals. It was found that 2-hexenal had pronounced synergistic effects on the pigment formation in the two-step TBA reaction of alka-2,4-dienals, suggesting that alk-2-enals had synergistic effects with alka-2,4-dienals. *t*-Butylhydroperoxide also increased the pigment formation from alka-2,4-dienals. The hydroperoxide may act as an oxidant for the intermediary adducts.

Limitation of the TBA test of oxidized lipids has been claimed since the test is nonspecific to malonaldehyde. It has been shown that the malonaldehyde content in oxidized lipids is much lower than expected (5-11). If the two-step reaction conditions are considered in the TBA test of lipid oxidation, the TBA test may be a useful method for measuring lipid oxidation products such as alka-2,4-dienals.

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Miss H. Takaoka and Y. Murakami provided technical assistance.

REFERENCES

1. Buege, J.A., and Aust, S.D. (1978) *Methods Enzymol. 52*, 302-310.
2. Sinnhuber, R.O., Yu, T.C., and Yu, T.C. (1958) *Food Res. 23*, 626-633.
3. Nair, V., and Turner, G.A. (1984) *Lipids 19*, 804-805.
4. Yu, L.W., Latriano, L., Duncan, S., Hartwick, R.A., and Witz, G. (1986) *Anal. Biochem. 156*, 326-333.
5. Frankel, E.N., and Neff, W.E. (1983) *Biochim. Biophys. Acta 754*, 264-270.
6. Hirayama, T., Yamada, N., Nohara, M., and Fukui, S. (1984) *J. Sci. Food Agric. 35*, 338-344.
7. Iwata, A., and Kikugawa, K. (1987) *Chem. Pharm. Bull. 35*, 5020-5023.
8. Csallany, A.S., Guan, M.D., Manwaring, J.D., and Addis, P.B. (1984) *Anal. Biochem. 142*, 277-283.
9. Esterbauer, H., Lang, J., Zarravec, S., and Slater, T.F. (1984) *Methods Enzymol. 105*, 319-328.
10. Bull, A.W., and Marnett, L.J. (1985) *Anal. Biochem. 149*, 284-290.

11. Kikugawa, K., Kato, T., and Iwata, A. (1988) *Anal. Biochem.*, in press.
12. Patton, S., and Kurtz, G.W. (1955) *J. Dairy Sci. 38*, 901.
13. Marcuse, R., and Johansson, L. (1973) *J. Am. Oil Chem. Soc. 50*, 387-391.
14. Pryor, W.A. (1980) in *Molecular Basis of Environmental Toxicity* (Bhatnagar, R.S., ed.), pp. 3-36, Ann Arbor Science Pub., Ann Arbor, MI.
15. Esterbauer, H., Cheeseman, K.H., Dianzani, M.U., Poli, G., and Slater, T.F. (1982) *Biochem. J. 208*, 129-140.
16. Witz, G., Zaccaria, A., Lawrie, N.J., Ferran, H.J., Jr., and Goldstein, B.D. (1985) *Toxicologist 5*, 919.
17. Kosugi, H., and Kikugawa, K. (1986) *Lipids 21*, 537-542.
18. Kosugi, H., Kato, T., and Kikugawa, K. (1987) *Anal. Biochem. 165*, 456-464.
19. Yagi, K. (1984) *Methods Enzymol. 105*, 328-331.

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Investigations of the Origin of the Furan Fatty Acids (F-Acids)

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The possible role of linoleic acid as a biogenetic precursor of the furan fatty acids (F-acids) was investigated in in vivo experiments in the rat, using a C_{19} analogue of linoleic acid and gas chromatography–mass spectrometry. No evidence of incorporation of this compound into the F-acids was found. Using an improved analysis procedure by converting F-acids into their tetrahydrofuran derivatives (enabling a separation from the large amounts of normal fatty acids), F-acids (F_3, F_4 and F_6) were detected in rat food, correcting earlier results.

Quantification of F-acid intake with food and excretion of furandicarboxylic acids in the urine, suggested the possibility that the F-acids are not produced de novo in the rat, but instead accumulate in tissue after nutritional intake.

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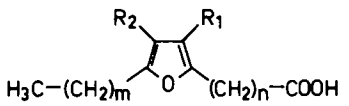
The F-acids are a group of furanoid fatty acids detected in extremely small quantities in a variety of animal (1–4) and, to a lesser extent, plant sources (5). The most common members of this family, the so-called F_0–F_6 [1 (a)–(g)], can be subdivided into a group bearing a propyl side chain (F_1 and F_4) and a group with a pentyl side chain (F_0, F_2, F_3, F_5 and F_6). Investigations of these compounds have focused on their synthesis (6–17) and on the isolation of new members of the family (18–23), but their biological significance, if any, remains undetermined.



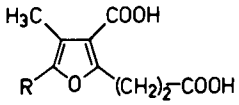
1

		m	n	R_1	R_2
a:	F_0	4	6	CH_3	CH_3
b:	F_1	2	8	CH_3	CH_3
c:	F_2	4	8	H	CH_3
d:	F_3	4	8	CH_3	CH_3
e:	F_4	2	10	CH_3	CH_3
f:	F_5	4	10	H	CH_3
g:	F_6	4	10	CH_3	CH_3
h:	[a]	4	9	CH_3	CH_3
i:	[a]	3	10	H	H
j:	[a]	3	10	CH_3	CH_3

[a]Synthetically produced unnatural furan fatty acids.

SCHEME 1

Relatively little information is available on the biogenesis of these compounds. Schlenk et al. (24) tried to incorporate [^{14}C]acetate into the F-acids in in vivo experiments with fish. However, the only detectable incorporation occurs via chain extension of unlabeled F-acid metabolites. It has also been shown that the F-acids may be further degraded to the urofuranic acids [2] in human feeding experiments, by correlating F-acid intake with urofuranic acid excretion (25). However, the origin of the F-acids themselves is still not known.



2

	R
a:	n-C_5H_{11}
b:	n-C_3H_7
c:	$C(O)CH_2CH_3$
d:	$CHOHCH_2CH_3$

SCHEME 2

It seems, on inspection of the structure of the F-acids, that the pathway presented in Scheme 1 provides a plausible route for these compounds. The initial step involves a lipoxygenase type reaction at C-13 to produce 13-peroxy-9,11-octadienoic acid. Subsequent ring closure is followed by double bond rearrangement and methylation to give the F-acids. Similar processes occur in prostaglandin biosynthesis (26), and the final methylation step has been demonstrated as possible in beef liver homogenates (27).

The feasibility of such a route is supported by results on the biosynthesis of the furanoacetylenic phytoalexin wyerone [3] (28,29). This compound has been shown to arise from acetate via a fatty acid-type precursor.

The lack of incorporation of acetate into the F-acid nucleus, as demonstrated by Schlenk for fish (24), may be rationalized by an assumption that fish cannot synthesize F-acids from acetate de novo, but may metabolize nutritionally derived linoleate and linolenate to F-acids. Alternatively, F-acids may not only be produced in mammals and fish, but may be accumulated in the body after additional intake with the food. We have attempted to resolve these questions by in vivo biosynthetic studies in rats and by elaborate examination of the rat food. The probable content of F-acid intake could be calculated from the amount of furan metabolites excreted.

MATERIAL AND METHODS

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). GC was carried out with a United

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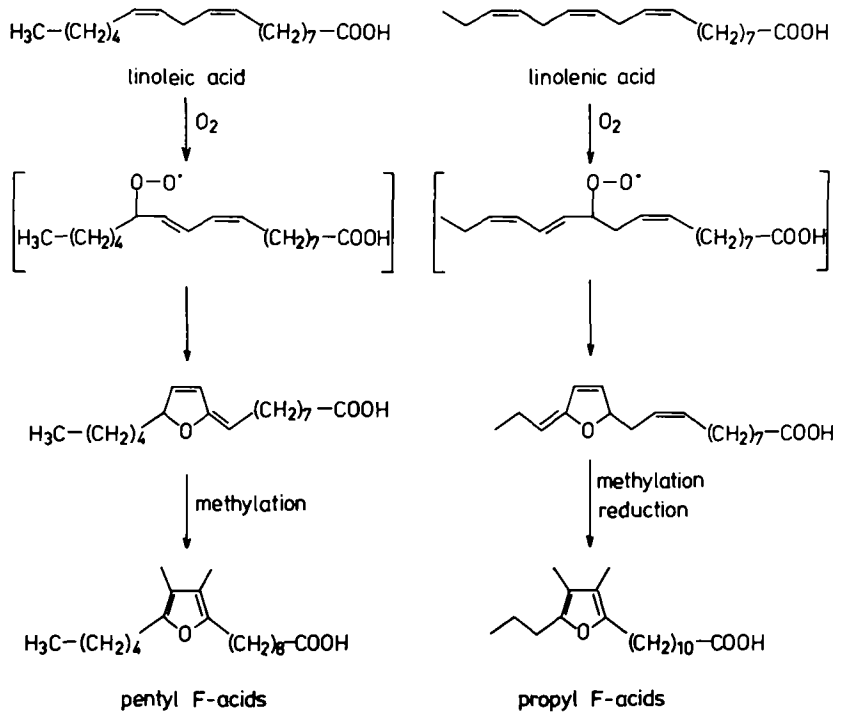
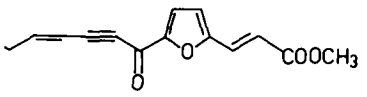
Abbreviations: GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; TLC, thin layer chromatography.



SCHEME 3. Putative biogenesis of the F-acids.



SCHEME 4

Technological Packard Model 438S with an FID, on a WCOT-glass capillary (30 m × 0.3 mm) OV-101 column, programmed from 150°C to 240°C at 2°C/min. The temperature of injector and detector was held at 270°C. Peak area integration was done by a Shimadzu C-R3A integrator. Carrier gas was hydrogen. The split ratio was 1:10.

GC–MS was performed on a Finnigan MAT 312 GC–MS system with a MAT-SS-300 data system, ionization energy 70 eV. The GC column was a 25 m × 0.3 mm i.d. OV-101 WCOT-glass capillary column. The carrier gas was helium (2 ml/min, the temperature program was the same as used for GC and the probes were injected in the splitless mode.

High performance liquid chromatography (HPLC) separation. HPLC was carried out using a Beckman 421 controller, two Beckman 114 M pumps, a Beckman 165 UV detector at 215 nm and a Beckman 156 RI detector. The column was a Serva Si100 (250 mm × 1.6 mm, 3 μm). The solvent was *n*-hexane/methyl-*t*-butyl ether (Merck, 97:3, v/v) at a flow rate of 1.7 ml/min^{-1}.

Synthesis of nonadeca-10,13-dienoic acid. The preparation of this compound from linoleic acid was as described by Marcel and Holman (30).

In vivo rat experiments. Four Wistar rats were kept on a linoleic acid-deficient diet (Altromin; No. C1058; 20 g/day) for 12 days. After this period, the food of three of the rats was supplemented with nonadeca-10,13-dienoic acid (40 mg/rat/day) for 33 days. The rats were killed and the liver, kidney, muscle, fat and blood fractions obtained. Typical work-up procedure for all fractions and the feces is as described for the liver fraction. To the liver fraction (15.6 g) was added [1j] (20 μg), methanol (60 ml) and chloroform (30 ml). The material was homogenized in a Waring blender at high speed for 4 min and then centrifuged at 3,000 rpm for 10 min. The supernatant was carefully removed and the homogenization and centrifugation repeated with chloroform (30 ml). Water (35 ml) and potassium chloride solution (0.88%, 60 ml) was added to the combined supernatants. After partition, the chloroform layer was evaporated to dryness under reduced pressure and the residue transesterificated with sodium methoxide in methanol (0.5 N, 30 ml) and tetrahydrofuran (15 ml) at 50°C for 1 hr under nitrogen. After cooling and acidification (concentrated HCl), most of the tetrahydrofuran was removed under reduced pressure and water (50 ml) was added. The solution was extracted with chloroform (3 × 50 ml), the chloroform removed under reduced pressure and the residue esterified with ethereal diazomethane.

Excess solvent and reagent wre removed in a stream of nitrogen. The residual oil was purified by column chromatography on SiO_2 (Merck, No. 7734, 50 g), collecting the fraction that eluted with cyclohexane/ethylacetate (98:2, v/v). This material was hydrogenated for 2 hr at 1.5 bar in the presence of a rhodium on alumina catalyst (5%, 20 mg). After filtration, the F-acid fraction was concentrated by thin layer chromatography (TLC) on SiO_2 (31), before final purification of the F-acid fraction by HPLC using *n*-hexane/methyl-*t*-butylether/2-propanol (97:3:0.05, v/v/v) as eluant. This material was used for GC–MS.

Examination of rat food. A portion of 20 g rat food (Altromin, No. C 1058) was crumbled in a grain mill (Braun, FRG). Internal standard [1j] 20 μg, methanol (160 ml), chloroform (80 ml) and water (60 ml) were added. The mixture was homogenized in a Waring blender at high speed for 4 min. After stirring the mixture for 3 hr, a second homogenization was done. Centrifugation at 3,000 rpm for 10 min gave a clear supernatant that was carefully removed. The residue was homogenized with chloroform (80 ml) and centrifuged. To the combined supernatants, potassium chloride solution (0.88%, 80 ml) was added. After partition, the lower chloroform layer was evaporated to dryness under reduced pressure. The remainder of the workup was as described for the rat liver fraction above.

Quantification of furandicarboxylic acids (4) in the rat urine. Twenty-four hr urine was collected with at least 0.3% NaN_3. Ten μg 3-carboxy-4-methyl-5-pentyl-furan-2-acetic acid was added as an internal standard. The urine was diluted with a 10-fold amount of cold (−20°C) acetone and left at −20°C overnight, to precipitate protein. The clear solution was decanted and the acetone was removed under reduced pressure. The residue was added, after dilution with 20 ml of water and acidification (concentrated HCl), to a C_{18} bonded-phase extraction column (Chromabond-C_{18}, 500 mg; Macherey & Nagel; D-5160 Düren, FRG; preconditioned with 5 ml methanol and 5 ml water) and drawn through the column under vacuum. The C_{18}-column was washed with 4 ml of 1 mol/l sodium acetate buffer (pH 5) and 4 ml water. Excess water was removed by suction and the final elution was achieved by aspirating 2 ml of methanol. Esterification of the methanol elute was done with excess ethereal diazomethane. After evaporation in the nitrogen stream, the residue was purified by column chromatography (150 mm × 8 mm) on 3 g SiO_2 (Merck No. 7734) in cyclohexane. Two fractions were collected. The first fraction with 20 ml cyclohexane/ethyl acetate (94:6, v/v), and a second fraction with 15 ml cyclohexane/ethyl acetate (8:2, v/v). The second fraction was concentrated to about 3 μl and 1 μl aliquots was used for GC and for GC–MS analyses. The amount of furandicarboxylic acids was calculated by peak area integration in comparison with the internal standard.

RESULTS AND DISCUSSION

The study of F-acid biosynthesis is complicated by the extremely small quantities of the compounds found in most living systems. Beef liver contains ca. 4.5 mg/kg of a mixture of F-acids (32), and a typical rat liver contains less than 10 μg of a similar mixture that will be discussed later. Separation of such extremely small quantities of F-acids from accompanying large amounts of fatty acids is neither practical nor possible even by the use of urea fractionation (3,32). Therefore, hydrogenation of F-acids to the corresponding tetrahydrofuran derivatives was done by using Rh/Al_2O_3 as a catalyst, because separation of tetrahydrofuran acids from fatty acids can be easily achieved by TLC (31). Thus, the F-acids are detected in the form of their tetrahydrofuran compounds in extremely small quantities (31), and analyzed structurally, using GC–MS. Moreover, all the F-acids naturally detectable in rat contain an even number of methylene groups in the carboxyl side chain [1, n = 6, 8, 10]. Consequently, if a C_{19} analog of linoleic acid containing an additional methylene group in the portion of the molecule between the carboxyl group and the first double bond is provided as precursor, the resultant F-acid after metabolism [1h] should contain a carboxyl side chain with an odd number of methylene groups (n = 9) and should be easily detectable and structurally distinguishable from naturally-occurring metabolites.

The substrate specificity of the enzyme system affecting the transformation from fatty acid to F-acid should, by analogy with investigations on prostaglandin biosynthesis (33,34), be tolerant of this chain elongation. For the prostaglandin, the initial sequence also involves a lipoxygenase-type reaction.

In vivo rat experiments. Before initiating in vivo rat-feeding experiments, it was necessary to confirm that F-acids were indeed present in the rat. Previous studies had shown the presence of the F-acid degradation products [4] in rat urine (35), but no investigation of the constituent F-acids had been made (36). A study of rat liver, kidney, muscle, fat and blood showed the F-acids F_3 [1d], F_4 [1e] and F_6 [1g] to be present in the approximate amounts shown in Table 1. It appears that F_3 and F_4 are the major F-acids present in the rat, as opposed to F_6 in beef.

Assuming that unsaturated fatty acids are precursors, this would suggest more efficient metabolism of a C_{18} precursor in the rat, as opposed to C_{20} in cattle, or more efficient chain elongation of a C_{18} derivative in cattle.

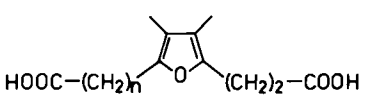
In the feeding experiments, rats were subjected to a diet deficient in linoleic acid for a period of two weeks before supplementation of the food with the C_{19} precursor was initiated. In this way, the daily intake of linoleic acid per rat was fixed at ca. 60 mg. After two weeks, the precursor (40 mg/day) was added to the food of each rat, and

TABLE 1

F-Acids Present in Rat

F-acid	Amount (μg/rat)[a]				
	Liver	Kidney	Muscle	Fat	Blood
F_3	—	1	4	18	—
F_4	—	3	4	12	—
F_6	1	—	7	1	—

[a]The values were determined by GC. Peak area was compared to the area of the unnatural F-acid [1j] added at a level of 20 μg to each sample. The results are estimated accurately to ± 20%.



n
a: 1
b: 2
c: 3
d: 4

SCHEME 5

feeding was continued for 33 days. At the end of this period, the liver, kidney, muscle, fat and blood fractions were once more examined. The results are presented in Table 2.

The natural F-acids, F_3 [**1d**], F_4 [**1e**] and F_6 [**1g**], are present in generally reduced quantities, but there is no evidence for the unnatural metabolite [**1h**]. In terms of the experimental design, this would suggest that, in the rat, linoleic acid is not a precursor of the F-acids. The natural metabolites are detectable at a level of ca. 0.1 μg, so even minimal conversion of the C_{19} precursor to [**1h**] should be apparent. Moreover, the metabolite [**1h**] has been synthesized in this laboratory ("unpublished results"), so that its retention index by GC is known, enabling extremely accurate analysis.

The fact that neither acetate (24) nor linoleate appear to be implicated in F-acid biosynthesis is surprising. However, an additional possibility is that the F-acids are not produced in animals, but instead originate in plants and are taken nutritionally into animal systems, whre they accumulate in various organs. However, earlier studies on fish food (37) and cattle food (36) have shown an absence of F-acids in these commodities. Methodology for F-acid detection has improved since these studies were made, and it could be that very small quantities of these metabolites were not detected. In the study of fish meal, detection is only claimed to be 0.2%. Recent work in this laboratory has confirmed the presence of F-acid transformation products in, e.g., soya bean oil ("unpublished results"), and they have been detected previously in seed oil (38). To clarify if previously undetectable amounts of F-acids may be introduced into the rats with their food, the rat food deficient in linoleic acid used in the above study was reexamined using the improved method for the detection of F-acids by hydrogenation to tetrahydrofuran acids. The results are presented in Table 3.

As can be seen, all three F-acids isolated from the rat are present in rat food in significant quantities. The amount of F_3 cannot be determined due to overlap of interfering peaks, but appears to be at least equal to the amount of F_4. It would seem possible, therefore, that the F-acids detected in the rat are not only produced in vivo, but also accumulate in the body via nutritional intake. It would explain the higher amounts of F-acids found in fatty tissue, where obviously no mechanism exists for their immediate removal from the body. In order to remove these F-acids [1], they are metabolized to dicarboxylic acids [4], which are then excreted in the urine.

TABLE 2

F-Acids Present in Rats Fed Food Supplemented with [1h] for 33 Days

F-acids	Amount (μg per rat)[a]				
	Liver	Kidney	Muscle	Fat	Blood
F_3	2	7	7	3	—
F_4	3	3	—	11	—
F_6	1	—	7	1	—

[a]The values were determined by GC. Peak area was compared with the area of the unnatural F-acid [1j] added at a level of 20 μg to each sample. The results are estimated accurately to ±20%.

TABLE 3

F-Acids Present in Rat Food

F-acids	Amount (μg/100$_g$)[a]	Daily intake per rat
F_3	*b*	*c*
F_4	15	3 μg ≙ 8.8 nmol
F_6	25	5 μg ≙ 13.6 nmol

[a]The values were determined by GC. Peak area was compared with the area of the unnatural F-acid [1j] added at a level of 20 μg to each sample. The results are estimated accurately to ±20%.

[b]Detectable, but not quantifiable because of overlapping peaks.

[c]Not determinable.

TABLE 4

Furandicarboxylic Acids in Rat Urine

Compound	Daily excretion per rat[a]
4b	1.3 μg = 5.0 nmol
4d	3.0 μg = 10.2 nmol

[a]The values determined by GC. Peak area was compared with the area of 3-carboxy-4-methyl-5-pentyl-furan-acetic acid added at a level of 10 μg to each sample. The results are estimated accurate to ±10%.

To elucidate the quantity of F-acids delivered by the food and to compare it with the quantity originating from de novo synthesis, it is necessary to quantify the urinary metabolites of the F-acids. The results are presented in Table 4. In addition, the feces were examined, but no F-acids could be detected. The amount of F-acids found in the rat food is sufficient to explain the origin of the amount of furandicarboxylic acid [**4b,4d**] in the rat urine, and the origin of the F-acids in the tissue.

Therefore, there is no need to assume a de novo synthesis of F-acids by the rat. This would account for the failure of biosynthetic experiments in both rat and fish.

Metabolism of supplementary F-acids to furandicarboxylic acids in rats has been shown to be relatively fast (35), thus explaining the low quantities of these compounds in organs such as liver and kidneys, but metabolism in fish is much slower (24), which accounts for the correspondingly larger quantities of F-acids found in this species.

F-acids present in rat tissue originate, therefore, from rat food and are metabolized to furandicarboxylic acids found in the rat urine.

Obviously, additional work, including a reexamination of different food, is necessary to further elucidate the origin of F-acids in other organisms. This is presently underway.

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REFERENCES

1. Glass, R.L., Krick, T.P., and Eckhardt, A.E. (1974) *Lipids 9,* 1004-1008.
2. Glass, R.L., Krick, T.P., Olson, D.L., and Thorson, R.L. (1977) *Lipids 12,* 828-836.
3. Glass, R.L., Krick, T.P., Sand, D.M., Rahn, C.H., and Schlenk, H. (1975) *Lipids 10,* 695-702.
4. Scrimgeour, C.M. (1977) *J. Am. Oil Chem. Soc. 54,* 210-211.
5. Hasma, H., and Subramaniam, A. (1978) *Lipids 13,* 905-907.
6. Elix, J.A., and Sargent, M.V. (1968) *J. Chem. Soc. (C),* 595-596.
7. Crundwell, E., and Cripps, A.L. (1973) *Chem. Phys. Lipids 11,* 39-42.
8. Lie Ken Jie, M.S.F., and Lam, C.H. (1977) *Chem. Phys. Lipids 20,* 1-12.
9. Lie Ken Jie, M.S.F. and Lam, C.H. (1977) *Chem. Phys. Lipids 19,* 275-287.
10. Lie Ken Jie, M.S.F., and Lam, C.H. (1978) *Chem. Phys. Lipids 21,* 275-287.
11. Rahn, C.H., Sand, D.M., Wedmid, Y., and Schlenk, H. (1979) *J. Org. Chem. 44,* 3420-3423.
12. Gunstone, F.D., and Wijesundera, R.C. (1979) *Chem. Phys. Lipids 24,* 193-208.
13. Rahn, C.H., Sand, D.M., Krick, T.P., Glass, R.L., and Schlenk, H. (1981) *Lipids 16,* 360-364.
14. Lie Ken Jie, M.S.F., and Ahmad, F. (1983) *J. Am. Oil Chem. Soc. 60,* 1783-1785.
15. Kern, W., and Spiteller, G. (1985) *Liebigs Ann. Chem.,* 1168-1174.
16. Pfordt, J., Thoma, H., and Spiteller, G.)1981) *Liebigs Ann. Chem.,* 2298-2308.
17. Lie Ken Jie, M.S.F. (1981) *Methods Enzymol. 72,* 443-471.
18. Gunstone, F.D., Wijesundra, R.C., and Scrimgeour, C.M. (1978) *J. Sci. Food Agric. 29,* 539-550.
19. Groweiss, A., and Kashman, Y. (1978) *Experientia 34,* 299.
20. Lie Ken Jie, M.S.F., and Sinha, S. (1981) *Phytochemistry 20,* 1863-1866.
21. Ota, T., and Takagi, T. (1983) *Bull. Fac. Fish. Hokkaido Univ. 34,* 88-92.
22. Okajima, H., Ishii, K., and Watanabe, H. (1984) *Chem. Pharm. Bull. 32,* 3281-3286.
23. Schödel, R., and Spiteller, G. (1987) *Lisbigs Ann. Chem.,* 459-462.
24. Sand, D.M., Glass, R.L., Olson, D.L., Pike, H.M., and Schlenk, H. (1984) *Biochim. Biophys. Acta 793,* 429-434.
25. Schödel, R., Dietel, P., and Spiteller, G. (1986) *Liebigs Ann. Chem.,* 127-131.
26. Bergström, S. (1967) *Science 157,* 382-391.
27. Dietel, P. (1987) Ph.D. Thesis, Universität Bayreuth, Bayreuth, FRG.
28. Cain, R.O., and Porter, A.E.A. (1979) *Phytochemistry 18,* 322-323.
29. Al-Douri, N.A., and Dewick, P.M. (1986) *Z. Naturforsch. 41C,* 34-38.
30. Marcel, Y.L., and Holman, R.T. (1968) *Chem. Phys. Lipids 2,* 173-182.
31. Puchta, V., and Spiteller, G. (1988) *Liebigs Ann. Chem.,* 25-28.
32. Schödel, R. (1986) Ph.D. Thesis, Universität Bayreuth, Bayreuth, FRG.
33. van Dorp, D.A., Beerthuis, R.K., Nugteren, D.H., and Vonkeman, H. (1964) *Biochim. Biophys. Acta 90,* 204-207.
34. Bergström, S., Danielsson, H., Klenberg, D., and Samuelsson, B. (1964) *J. Biol. Chem. 239,* PC 4006-4008.
35. Sand, D.M., Schlenk, H., Thoma, H., and Spiteller, G. (1983) *Biochim. Biophys. Acta 751,* 455-461.
36. Bauer, S., and Spiteller, G. (1985) *Helv. Chim. Acta 68,* 1635-1638.
37. Gunstone, F.D., and Wijesundra, R.C. (1978) *J. Sci. Food Agric. 29,* 28-32.
38. Morris, L.J., Marshall, M.O., and Kelly, W. (1966) *Tetrahedron Lett.,* 4249-4253.

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Localization of Long-Chain Fatty Acids and Unconventional Sterols in Spherulous Cells of a Marine Sponge[1]

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The first direct evidence is provided for the presence of unconventional lipids in a particular subcellular membrane system of a sponge. Spherulous cells were isolated from the variety of cell types present in the marine sponge *Aplysina fistularis* by density gradient centrifugation. Spherulous cell plasma membrane was subsequently isolated by cell rupture followed by differential centrifugation and sucrose, or Percoll, density gradient ultracentrifugation. Plasma membrane isolates were identified and assessed for purity using [^{3}H]concanavalin A plasma membrane marker, sodium dodecyl sulfate polyacrylamide gel electrophoresis and ratios of protein, sterol and phosphate. Plasma membrane isolates could not be assessed for purity by traditional enzymatic means. Spherulous cell plasma membrane was found to contain unusual lipids, including long-chain (C_{24}–C_{30}) fatty acids (16.8–27.2%) and unconventional 26-alkylated sterols (66.4–72.6%), in addition to more conventional fatty acids and sterols. Spherulous cell intracellular membranes were also found to contain long-chain fatty acids and unconventional sterols, although the relative importance of these unusual lipids apparently varies between intracellular membranes, with some containing approximately 50% long-chain acids.
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Unusual lipids, including long-chain fatty acids (LCFA) (C_{24}–C_{30}) and unprecedented sterols, have been identified from mixed membrane isolates of whole marine sponges, raising intriguing questions about the structure and function of sponge membranes, as such lipid content contrasts with the shorter-chain fatty acid (C_{14}–C_{22}) and simple sterol (cholesterol) content of other animal cell membranes (1–3). Since the mixed membrane isolates examined to date have consisted of a mixture of subcellular membranes from the entire diversity of cell types present within the adult sponge, the lipid composition of any specific sponge subcellular membrane system is unknown. Moreover, it is also unknown whether LCFA co-occur with unconventional sterols in the cellular membranes of these animals. Such knowledge is critical to an understanding of sponge-membrane structure and function.

Therefore, the purpose of the present study was to isolate and chemically characterize the lipid composition of a specific subcellular membrane (plasma membrane) from one type of sponge cell that was known to contain both unusual fatty acids and sterols. Spherulous cells of the marine demosponge *Aplysina fistularis* were chosen for this study for the following reasons: (A) In our preliminary studies, spherulous cells were found to contain large quantities of LCFA extending to at least C_{30}-chain lengths (unidentified fatty acids with equivalent chain lengths [ECL] corresponding to C_{31}–C_{33} also were detected) and 26-alkylated sterols (Fig. 1) as previously reported for the whole sponge (4–6). (B) Spherulous cells in this sponge are present in large numbers and can be

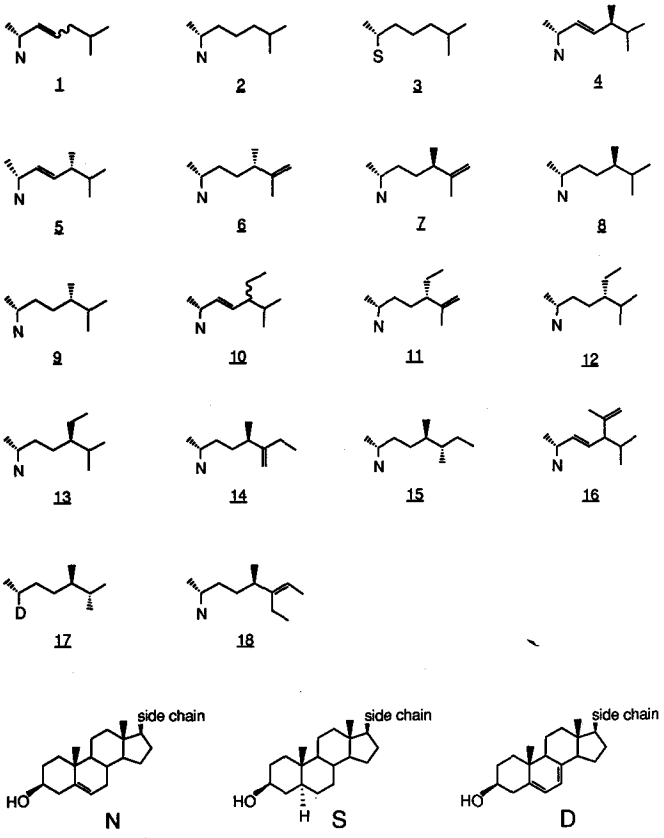

FIG. 1. Major sterols (≥0.5%) present in *Aplysina fistularis*: E/Z-cholesta-5,22-dien-3β-ol (1); cholest-5-en-3β-ol (cholesterol) (2); 5α-cholestan-3β-ol (3); E-(24S)-methylcholesta-5,22-dien-3β-ol (crinosterol) (4); E-(24R)-methylcholesta-,22-dien-3β-ol (brassicasterol) (5); (24S)-methylcholesta-5,25-dien-3β-ol (codisterol) (6); (24R)-methylcholesta-5,25-dien-3β-ol (epicodisterol) (7); (24R)-methylcholest-5-en-3β-ol (8); (24S)-methylcholest-5-en-3β-ol (9); (24E)-ethylcholesta-5,22-dien-3β-ol (10); (24S)-ethylcholesta-5,25-dien-3β-ol (clerosterol) (11); (24S)-ethylcholest-5-en-3β-ol (12); (24R)-ethylcholest-5-en-3β-ol (13); (24R)-24,27-dimethylcholesta-5,25-dien-3β-ol (25-dehydroaplysterol) (14); (24R,25S)-24,26-dimethylcholest-5-en-3β-ol (aplysterol) (15); (24S[or R])-isopropenylcholesta-5,22-dien-3β-ol (16); (24R,25S)-24,26-dimethylcholesta-5,7-dien-3β-ol (7-dehydroaplysterol) (17); E-(24R)-24,26,27-trimethylcholesta-5,25-dien-3β-ol (verongulasterol) (18).

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Abbreviations: BSA, bovine serum albumin; CMF-ASW, calcium- and magnesium-free artificial sea water; [^{3}H]con A, [^{3}H]concanavalin A; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid; HM, homogenizing medium; LB, labeling buffer; LCFA, long-chain fatty acid; P1, P2 and P3, pellets obtained by respective centrifuging at 1075, 15,000 and 100,000 × *g*; PUFA, polyunsaturated fatty acid; SCFA, short-chain fatty acid; SDS, sodium dodecyl sulfate; ECL, equivalent chain length; GC, gas chromatography; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MS, mass spectrometry; TLC, thin layer chromatography.

readily and efficiently isolated from other sponge cells and abundant matrix microorganism symbionts (7). (C) Spherulous cells are comparatively large (ca. 10 μm dia.), highly refractile cells that can be readily differentiated from other sponge or symbiont cells due to the presence of large organelles termed spherules (1-3 μm dia.) that contain distinctive, yellow-colored, globular inclusions (8); thus, spherulous cells can be easily observed unaided by light microscopy for rapid verification of spherulous cell isolate purity and cell rupture. (D) Apart from spherules, spherulous cells contain few other intracellular organelles with membranes that could contaminate plasma membrane isolates from these cells (7,9). Finally, the basic histology and behavior of these cells is well known (8,9), including the fact that spherules contain brominated secondary metabolites (7), possibly for use in chemical defense or offense (10). Also abundant in the whole sponge are bacterial symbionts and archaeocyte, pinacocyte and choanocyte sponge cells. Research is currently underway in our laboratory to isolate these cell types.

This study is the first to apply traditional methods of density gradient ultracentrifugation for the purification of plasma membrane from sponge cells. The traditional enzyme assay method for characterization of subcellular fractions, however, could not be considered conclusive for this poorly understood animal system (as discussed further in Results). Therefore, additional methods for the detection of spherulous cell plasma membrane and assessment of its purity were also employed, including radioactive labeling ([^{3}H]concanavalin A) of spherulous cell plasma membrane prior to cell rupture, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and ratios of protein, phosphate and sterol.

EXPERIMENTAL

Collection. Aplysina fistularis (Pallas, 1776) *(Verongia thiona, Verongia aurea, Spongia fulva)* (O. Verongida) was collected in March (Experiment 1) and June 1986 (Experiment 2), from the low intertidal (1-3 m below mean lower, low water) at Casa Cove, La Jolla, CA. Sponges were maintained in aerated seawater (6°C) for no longer than 3 days prior to use.

Chemicals and materials. Density gradient chemicals included Ficoll (Type 400 DL) and grade 1 sucrose (Sigma; St. Louis, MO), and Percoll (Pharmacia Inc.; Uppsala, Sweden). Other supplies were: concanavalin A (N-[acetyl-^{3}H] acetylated; 30-90 Ci/mmol; Pharmacia Inc.); methyl α-D-mannopyranoside (Sigma); AcrylAide and GelBond (FMC Corporation, Princeton, NJ); Aquasol (NEN Research Products universal cocktail); and Fiske-Subbarrow reducing agent (Sigma). Bovine serum albumin (BSA) and cholesterol (Sigma) were standards for protein and sterol quantification, respectively. SDS gel electrophoresis standards (Sigma) consisted of 3 kit samples with molecular weight ranges of: 14-70K (product no. MW-SDS-70L), 30-200K (MW-SDS-200) and a prestained selection of 27-180K (MW-SDS-BLUE) proteins. Electron microscopy reagents, reference lipids and other chemicals and materials used in lipid analysis were those listed in Lawson et al. (2).

Tissue samples were held on ice throughout all procedures, unless otherwise stated.

Isolation of spherulous cells. Spherulous cells were isolated from whole sponge tissue according to methods modified after Thompson et al. (7). To dissociate cells, sponges (1 kg, wet wt) were cleaned of macroscopic debris and epibionts, cut into pieces (1 cm^3), washed in calcium-magnesium-free artificial seawater (CMF-ASW) (6 l, 10°C), left in CMF-ASW (25°C) for 1-2 hr and finally forced through cheesecloth. Residual tissue (300 g, wet wt) was retained for lipid analysis. To isolate spherulous cells, the dissociated cells (2 g, wet wt) were concentrated by centrifugation (650 × *g*/5 min/10°C; Sorvall RC-5 centrifuge, GSA rotor), resuspended in CMF-ASW/10°C to a total volume of 20 ml and placed on top of several discontinuous Ficoll gradients (10°C). The gradients were produced by layering from bottom to top the following concentrations of Ficoll in CMF-ASW in 40 ml transparent centrifuge tubes: 23.1% (10 ml), 20% (10 ml), 16.7% (5 ml) and 9.1% (5 ml). The gradients were centrifuged (2,000 rpm/5 min/25°C; International clinical benchtop centrifuge Model CL, rotor #211) and spherulous cells were recovered from the 23.1-20% Ficoll interface by aspiration with a truncated Pasteur pipette. Spherulous cells were washed free of Ficoll in CMF-ASW (250 ml; 600 × *g*/15 min/10°C), resuspended in CMF-ASW (1 ml) and applied to a second Ficoll gradient that consisted from bottom to top of 20% (10 ml), 16.7% (10 ml) and 9.1% (10 ml) Ficoll in CMF-ASW/10°C for further purification. Centrifugation was repeated as for the first gradient, and spherulous cells were collected from the 20-16.7% Ficoll interface and washed as before. Homogeneity of cell fractions was assessed by light and electron microscopy.

[^{3}H]Concanavalin A ([^{3}H]con A) labeling of plasma membrane. Spherulous cells (0.5 g, wet wt) isolated in June were: A) washed free of Ficoll in CMF-ASW/10°C, B) stabilized in 3.5% formaldehyde/CMF-ASW/10°C (20 min) to prevent reaggregation of dissociated cells (9,11), C) washed free of formaldehyde in CMF-ASW/10°C (3×) and finally incubated with [^{3}H]con A (200 nCi/ml) in labeling buffer (LB) (800 mM sorbitol/50 mM Tris-HCl/0.2 mM ethylene glycol bis[β-aminoethyl ether] N,N'-tetraacetic acid [EGTA]/1mM $MgCl_2$/1 mM $CaCl_2$/1 mM $MnCl_2$/BSA [3 g/ml]/CMF-ASW/10°C; pH 7.4; 5-10 ml) for 40 min on ice with gentle shaking every 10 min (12). Cells were centrifuged (2,000 rpm/25°C/5 min) and the supernatant was discarded. Pelleted cells were washed (3×) in LB lacking EGTA (150 ml) (12) and used for Experiment 2, as described below.

The saturation point of [^{3}H]con A labeling to spherulous cell plasma membrane was determined by a standard kinetic study involving subsampling at 0, 10, 30 and 60 min (13). The effects of Ficoll on labeling with [^{3}H]con A were examined by comparing the labeling efficiency in the absence and presence of Ficoll (30%). Possible internalization of the [^{3}H]con A label was investigated by incubating spherulous cells in 200 nCi/ml [^{3}H]con A in LB 10°C/40 min/pH 7.4 according to Sorimachi (13) and was determined to be the proportion of cell-associated radioactivity (lectin binding) remaining after removal of the externally-bound ligand by methyl α-D-mannopyranoside (13). Enrichment of [^{3}H]con A radioactivity was calculated as a ratio of the [^{3}H]-radioactive count per mg protein in subcellular isolates from Percoll gradients compared with that of parent [^{3}H]-labeled cells used in the

experiment (12). Data were corrected for nonspecific binding of [^{3}H]con A, which was assessed by conducting parallel assays with the haptan monosaccharide, methyl α-D-mannopyranoside (100 mM) being present either from t = 0 or t = 60 min, during the course of [^{3}H]con A labeling according to the methods of Cuatrecasas (14), Juliano and Li (15), Emerson and Juliano (16) and Chicken and Sharom (17).

Isolation of spherulous cell plasma membrane. Experiment 1. Subcellular fractionation of spherulous cells was modified after Lawson et al. (2). Spherulous cells were resuspended and homogenized in homogenizing medium (HM) (HM1 = 250 mM sucrose/50 mM Tris-HCl/5 mM EDTA/0.1 mM phenylmethylsulfonylfluoride/CMF-ASW/10°C, pH 7.4) with a Brinkman Polytron homogenizer (setting 1, 30 sec). The homogenate was filtered (0.5 × 0.1 mm mesh nylon) and subjected to successive centrifugation at 1075 and 15,000 × *g* (10 min/10°C; Sorvall RC-5 centrifuge, SS-34 rotor), and 100,000 × *g* (1 hr/4°C; Beckman L2-65B centrifuge, SW-28 rotor) to produce P1, P2 and P3 pellets (see Abbreviations) and a final supernatant, respectively. P1 and P2 pellets were each washed once in HM1 and lyophilized for lipid analysis. The P3 pellet was resuspended in buffer (8.5%, w/w = 250 mM sucrose/50 mM Tris-HCl/CMF-ASW/4°C; pH 7.4; 5 ml) and placed on top of a sucrose density gradient, which consisted of (from bottom to top): 45% (1.32 M, 5 ml), 42.8% (1.25 M, 7 ml), 37.7% (1.1 M, 7 ml), 34.1% (1.0 M, 7 ml), 30% (0.88 M, 7 ml) sucrose/50 mM Tris-HCl/CMF-ASW/4°C with respective densities of 1.21, 1.19, 1.17, 1.16, 1.13 and 1.03 g/ml at 0°C (18). The sucrose gradient was centrifuged (131,000 × *g*/2 hr/4°C; Beckman L2-65B ultracentrifuge, SW-28 rotor) and visually-distinct fractions were collected by aspiration with a Pasteur pipette.

Experiment 1. [^{3}H]Con A-labeled spherulous cells (described above) were resuspended and homogenized (HM2 = 800 mM sorbitol/50 mM Tris-HCl/1 mM $MgCl_2$/1 mM $MnCl_2$/1 mM $MnCl_2$/5 mM β-mercaptoethanol/CMF-ASW/10°C; pH 7.4; 5 ml; hand-held Biospec Biohomogenizer; low speed—10,000 rpm/2 min; on ice) (12,13,19) and fractionated according to methods modified after Fauvel et al. (12). The homogenate was centrifuged (1,000 × *g*/15 min/4°C; Sorvall RC-5 centrifuge, SS-34 rotor), and the supernatant (containing subcellular organelles and microsomal membranes) was mixed with twice-concentrated HM2 in Percoll/CMF-ASW (6:12:10.32:1.68, v/v/v/v) at pH 7.4 and centrifuged (79,000 × *g*/15 min/4°C; Beckman L2-65B ultracentrifuge, SW-28 rotor) to produce a continuous Percoll gradient according to modified methods of Fauvel et al. (12). Fractions of 2 ml were successively collected from the top of the gradient with Pasteur pipettes. Fractions 2–4, which characteristically contain subcellular membranes (12), were combined and mixed with twice-concentrated HM2/Percoll (6:12:10.32, v/v/v) (pH 9.6, adjusted with 1 M NaOH), with a final pH adjustment to 9.6. This mixture was centrifuged (79,000 × *g*/15 min/4°C) and fractions were collected for analysis from the top of the gradient with Pasteur pipettes.

Microscopy. Foramalin-fixed, spherulous cell isolates were examined by light microscopy, utilizing toluidine blue as a staining reagent. Samples of whole sponge, spherulous cells and subcellular isolates were examined by electron microscopy as in Lawson et al. (2). The purity of the mixed membrane isolate, in terms of it being free from subcellular lipidic contaminants such as small vacuoles, lipid droplets and osmiophilic granules (that are characteristically found in spherules), was determined by electron microscopy (2).

Enzyme assays. A coupled enzyme assay, in which formation of ADP by ATPase is coupled to NADH oxidation with the intermediate enzymes pyruvate kinase and lactic dehydrogenase and with the intermediate substrate phosphoenolpyruvate present in excess, was employed to determine membrane-associated Na^+K^+-ATPase activity in aliquots of subcellular isolates according to the methods of Scharschmidt et al. (20).

Determination of radioactivity. Samples of [^{3}H]con A-labeled spherulous cells and subcellular fractions from Experiment 2 were digested in 1 ml household bleach to reduce quenching and the mixture was sonicated (1 min) before the addition of scintillation fluid (Aquasol) to aid in suspension of subcellular tissue. Suspensions were shaken immediately prior to counting radioactivity (Beckman LS 7500 microprocessor controlled liquid scintillation system with external standardization and quench correction).

SDS-Polyacrylamide gel electrophoresis. Samples of whole spherulous cells, homogenates, P1 and P2 pellets, membrane bands and intermediate fractions from Experiment 1, and membrane bands and intermediate fractions from Experiment 2, were analyzed for protein content by SDS-polyacrylamide gel electrophoresis (PAGE) (21). Samples containing 10–20 μg protein were solubilized in buffer (55 mM Tris-HCl/1.8% SDS/8.8% glycerol) (1 part protein:1 part buffer), to which 0.002% bromophenol blue tracking dye was added, at 100°C/2 min. Samples (50 μl) were applied to SDS-polyacrylamide-discontinuous-slab gels containing 10% AcrylAide (4.5% stacking gel, 7% running gel/backed with GelBond) (22,23) and run at 27 mA. Gels were stained with Coomassie Brilliant Blue R and/or silver stained. To detect nonspecific staining, controls of buffers and suspension media were simultaneously run.

Chemical analyses. Lipids were extracted from lyophilized (Experiment 1) or wet/frozen (Experiment 2) tissue, cells and subcellular fractions and were examined by thin layer chromatography (TLC) for lipid class content according to Lawson et al. (2).

The majority of the total lipid extract from the Experiment 1 whole sponge sample and the Experiment 2 whole spherulous cell isolate (60 mg) was separated by column chromatography (7 × 0.7 cm dia. column; SIL-R 100–300 mesh silicic acid, Sigma Co.) into fractions that usually contain triacylglycerols and sterol esters (Fraction 1), free sterols and diacylglycerols (Fraction 2), glycolipids (Fraction 3) and phospholipids (Fraction 4). Fraction 1 was eluted with hexane/ether (2:1, v/v, 20 ml), Fraction 2 with acetone (40 ml), Fraction 3 with methanol (50 ml) and Fraction 4 with chloroform/methanol (2:1, v/v, 20 ml). The composition of eluates was monitored by TLC that utilized codeveloped reference compounds and spray reagents according to Lawson et al. (2). The remainder of the total lipid extracts were used for total lipid fatty acid and total sterol analysis.

Fatty acid composition of all samples, including Experiment 1 whole sponge sample total lipid and lipid

Fractions 1-4, Experiment 1 spherulous cell subcellular fractions and Experiment 2 whole sponge tissue, spherulous cells and spherulous cell subcellular fractions, was determined by temperature-programmed gas chromatography (GC) as described in Lawson et al. (2). Major (≥0.5%) individual fatty acid methyl esters were identified by GC-mass spectrometry (MS) of N-acylpyrrolidide derivatives of the acids (24) according to Lawson et al. (2).

Sterols from Experiment 1 whole sponge tissue Fraction 1 sterol esters were obtained by: A) dissolution in methanol/water (99.5:0.5, v/v), B) addition of KOH (15 mg), C) heating (95°C/5 min) with stirring, D) concentration in vacuo, E) extraction in hexane and F) purification through a Pasteur pipette column of silica gel with hexane/ether (2:1, v/v). Total sterols were obtained from the Experiment 1 whole sponge tissue and subcellular fractions, and the Experiment 2 whole sponge tissue, spherulous cells and spherulous cell subcellular fractions total lipid extracts, according to the same methods. Free sterols were isolated from Experiment 1 whole sponge tissue Fraction 2 by column chromatography in hexane/ether (2:1, v/v). Sterol composition of these samples, for sterols present in amounts of >0.5% of the total, was determined by high performance liquid chromatography (HPLC), GC and GC-MS, as in Lawson et al. (3).

Phospholipid composition was determined by two-dimensional TLC of Experiment 2 whole spherulous cells and P1 pellet, and Experiment 1 whole sponge tissue, P1 and P2 pellets, as described in Lawson et al. (2). Insufficient sample was available for phospholipid analysis of membrane isolates.

Total sterol, protein and phosphorus was quantified in subsamples of A) Experiment 2 spherulous cells, B) Experiment 2 Mixed Membrane Isolate, C) Experiment 1 spherulous cell P1 fraction, D) Experiment 1 intracellular membrane bands 2 and 3 and E) plasma membrane isolates from both experiments. Free sterol content was quantified using the fluorometric procedure of Heider and Boyett (25), with cholesterol as standard. Total protein was determined using a modified Lowry procedure (26), with BSA as standard. Total phosphorus was determined according to Johnson (27), using monobasic potassium phosphate as standard. Inorganic phosphate obtained after digestion was estimated by the high temperature Fiske-Subbarow method as in Bartlett (28). Phospholipid content was estimated assuming 770 g/mol as the average molecular weight of the phospholipids (29).

RESULTS

Isolation of spherulous cells. Intact *A. fistularis* spherulous cells were isolated at greater than 98% purity by volume, as determined by electron microscopy. The isolated spherulous cells were observed to contain multiple lipophilic spherules (2-7 μm), few mitochondria and small amounts of endoplasmic reticulum, in accordance with previous results (7,9). It was necessary to repeat the cell fractionation procedure (with a reduced number of density layers) on the primary spherulous cell isolate to achieve such purity, however, as the primary spherulous cell isolate was contaminated by noticeable aggregations of smaller, weakly refringent cells and organic debris.

[3H]Con A-labeling of plasma membrane. Maximum radioactive labeling of the spherulous cell plasma membrane with the plant lectin [3H]con A was achieved after 30-40 min, with 20% labeling efficiency; 88% of the [3H]con A-labeling at 0-40°C was found to be due to specific membrane binding. High concentrations of Ficoll (30%) diminished labeling efficiency by ca. 10%, but residual Ficoll that remained after washing spherulous cells did not have a significant effect on labeling. Less than 6% of the [3H]con A-radioactive label was internalized under the conditions used; this small amount of internalization of the label, although demanding consideration, did not significantly influence the experiment.

Isolation of spherulous cell plasma membrane. Experiment 1. Homogenization and differential centrifugation of unlabeled spherulous cells produced a relatively pure P3 mixed-membrane isolate, as evidenced by the almost exclusive presence of membrane vesicles in electron micrographs. Substantial P1 and P2 isolates were also produced that included many intact, membrane-bounded spherules, suggesting that these intracellular organelles largely were not disrupted during homogenization.

Subsequent centrifugation of the P3 mixed membrane pellet on a sucrose density gradient resulted in three discrete cloudy-white bands and a very small brown-colored pellet. The uppermost band, Band 1, contained most visible material and banded isopycnically at a sucrose density of 1.03-1.13 g/ml. The sedimentation density of this band corresponds to that reported for plasma membranes (18) in accordance with the proportionately larger size of this band; hereafter, this band will be referred to as the Plasma Membrane Band 1. Band 2 was faint and occurred at a density of 1.16-1.17 g/ml, and Band 3 was less faint and occurred at 1.17-1.19 g/ml. The sedimentation densities of Bands 2 and 3 correspond to those reported for endoplasmic reticulum and mitochondria, respectively (18), although spherule membranes were likely also included in Band 2 and/or 3; hereafter these bands will be referred to as Intracellular Membrane Bands 2 and 3. The pellet mainly consisted of membrane-bound debris.

Because we could not isolate [3H]con A-labeled plasma membrane on a sucrose density gradient because of competition for the radioactive label between sucrose and the carbohydrate residues in the plasma membrane, the following method was used for this purpose.

Experiment 2. Homogenization and differential centrifugation of [3H]con A-labeled spherulous cells produced a supernatant fraction that contained microsomal membranes. Density gradient ultracentrifugation of this supernatant on Percoll at pH 7.4 resulted in a low-density, cloudy-white band at 4-10 ml (as measured from the top), a region expected to accumulate a mixture of subcellular membranes (12); the tritiated radioactivity of this band, hereafter referred to as the Mixed Membrane Isolate, was enriched 2.8 times. A second discreet band, which banded at a higher density (14-16 ml) and was light brown in color, consisted of unidentified cellular material with a 1.2 times enrichment in radioactivity; hereafter this band will be referred to as the Unidentified Cellular Material Isolate. A pellet consisting of spherules and unidentifiable cellular debris also possessed a relatively high radioactivity level.

Upon recentrifugation of the Mixed Membrane Isolate on Percoll at pH 9.6, a single band was observed at 4-10 ml, the same position as the Mixed Membrane

Isolate on the first Percoll gradient. This suggests that the Mixed Membrane Isolate of the previous centrifugation (pH 7.4) was largely plasma membrane, in accordance with the observation of only a slight enrichment in radioactivity of this band after recentrifugation at pH 9.6. Because some purification of the plasma membrane isolate was achieved, nonlabeled intracellular membranes must have been present on the gradient; however, they were not visually apparent, which is probably due to the small amount present. This band, hereafter referred to as the Plasma Membrane Isolate, was enriched 3.1 times in radioactivity, indicating slightly greater purity.

Enzyme assays. Attempts to measure levels of membrane-associated Na^+ K^+-ATPase activity in the above membrane isolates, spherulous cells and related whole sponge tissues were unsuccessful, eliminating the most widely used method for characterization of subcellular isolates and determination of membrane purity. The presence of cytotoxic, brominated natural products in the spherulous cells of *A. fistularis* (7) may act to inhibit this enzyme in vitro as such compounds are powerful inhibitors of Na^+ K^+-ATPase (30,31). An alternative enzyme system, 5′-nucleotidase, which is also frequently used to characterize plasma membranes (32), similarly yielded poor levels of activity; hence, further investigation of enzyme activities was discontinued. Although disappointing, these results were not surprising, as the enzyme composition of sponge cell membranes has not been characterized and there are few studies that report on sponge enzymatic activity. Subsequent unpublished experiments by Lucinda Cordeiro in our laboratory have also failed to utilize the following enzymes as potential sponge membrane markers: K^+-stimulated ATPase, acid phosphatase, NADPH cytochrome c reductase, NADH cytochrome c reductase, which were assayed in the sponge *Microciona prolifera*.

SDS-PAGE. SDS-PAGE of spherulous cell homogenates and membrane fractions obtained from Experiments 1 and 2 produced protein profiles containing 10–14 different bands. The principal bands occurred at 225 K, 175 K, 109 K, 101 K, 93 K, 66.2 K, 64.9 K, 48 K and 46.5 K, with a broad unresolved cluster of bands consisting of several proteins between 33.4–37 K, 24 K, 15 K and 10 K.

Three proteins appeared characteristic of particular subcellular isolates. A high molecular weight protein (225 K) was exclusively present in those isolates that were enriched in plasma membrane (Plasma Membrane Band 1 of Experiment 1 and both the Mixed Membrane Isolate and the Plasma Membrane Isolate of Experiment 2). In contrast, proteins of 175 K and 93 K only were exclusively present in the Intracellular Membrane Bands 2 and 3 of Experiment 1 and the Unidentified Cellular Material Isolate of Experiment 2.

Chemical analyses—whole sponge lipid content. Total lipid extracts of whole sponges collected in March (Experiment 1) and June (Experiment 2) were yellow/brown and yielded identified fatty acids to 30 carbons and unconfirmed fatty acids to C_{31}–C_{33} carbons (Table 1), with two main groupings of fatty acids constituting greater

TABLE 1

The Fatty Acids of *A. fistularis* According to Equivalent Chain Length, as Determined by Gas Chromatography

Peak number	ECL	Identity	Peak number	ECL	Identity
1	14.00	C14:0	33	18.90	C19:0 cyclo,11-12
2	14.39	C15:1br	34	19.00	C19:0
3	14.44	C15:1br	35	19.40	C20:4 *cis* 5,8,11,14
4	14.53	C15:1br	36	19.47	C20:4 *cis* 6,9,12,15
5	14.62	C15:0br	37	19.60	C20:2 *cis* 5,9
6	14.71	C15:0br	38	19.83	C20:1
7	15.00	C15:0	39	20.00	C20:0
8	15.08	unknown	40	20.64	unknown
9	15.39	C16:1br	41	20.75	unknown
10	15.46	C16:1br	42	21.35	unknown
11	15.63	C16:0br	43	22.00	C22:0
12	15.72	C16:0br	44	23.24	C24:5
13	15.77	C16:1	45	23.34	unknown
14	15.82	C16:1 *cis* 9	46	24.64	C25:0br
15	15.91	C16:1	47	24.75	C25:0br
16	16.00	C16:0	48	24.84	unknown
17	16.42	C17:1br	49	25.48	unknown
18	16.45	C17:1br	50	25.65	C26:2 *cis* 5,9
19	16.63	C17:0br	51	26.06	unknown
20	16.72	C17:0br	52	26.40	unknown
21	16.89	C17:0 cyclo	53	26.65	C27:0br
22	17.00	C17:0	54	26.99	C27:0
23	17.25	C16:0,2-OH	55	27.12	unknown
24	17.41	C18:1br	56	27.49	C28:3 *cis* 5,9,21
25	17.61	C18:2 *cis* 5,9	57	27.57	C28:3 *cis* 5,9,23
26	17.77	C18:1 *cis* 9	58	28.09	C28:2 *cis* 5,9
27	17.82	C18:1	59	28.68	C29:3 *cis* 5,9,23
28	17.92	C18:1	60	29.61	C30:3
29	18.00	C18:0	61	30.00	C30:0
30	18.19	unknown	62	30.13	unknown
31	18.40	C18:0br-11	63	32.76	unknown
32	18.60	unknown			

Abbreviations: br, branched (position); cyclo, cyclopropyl.

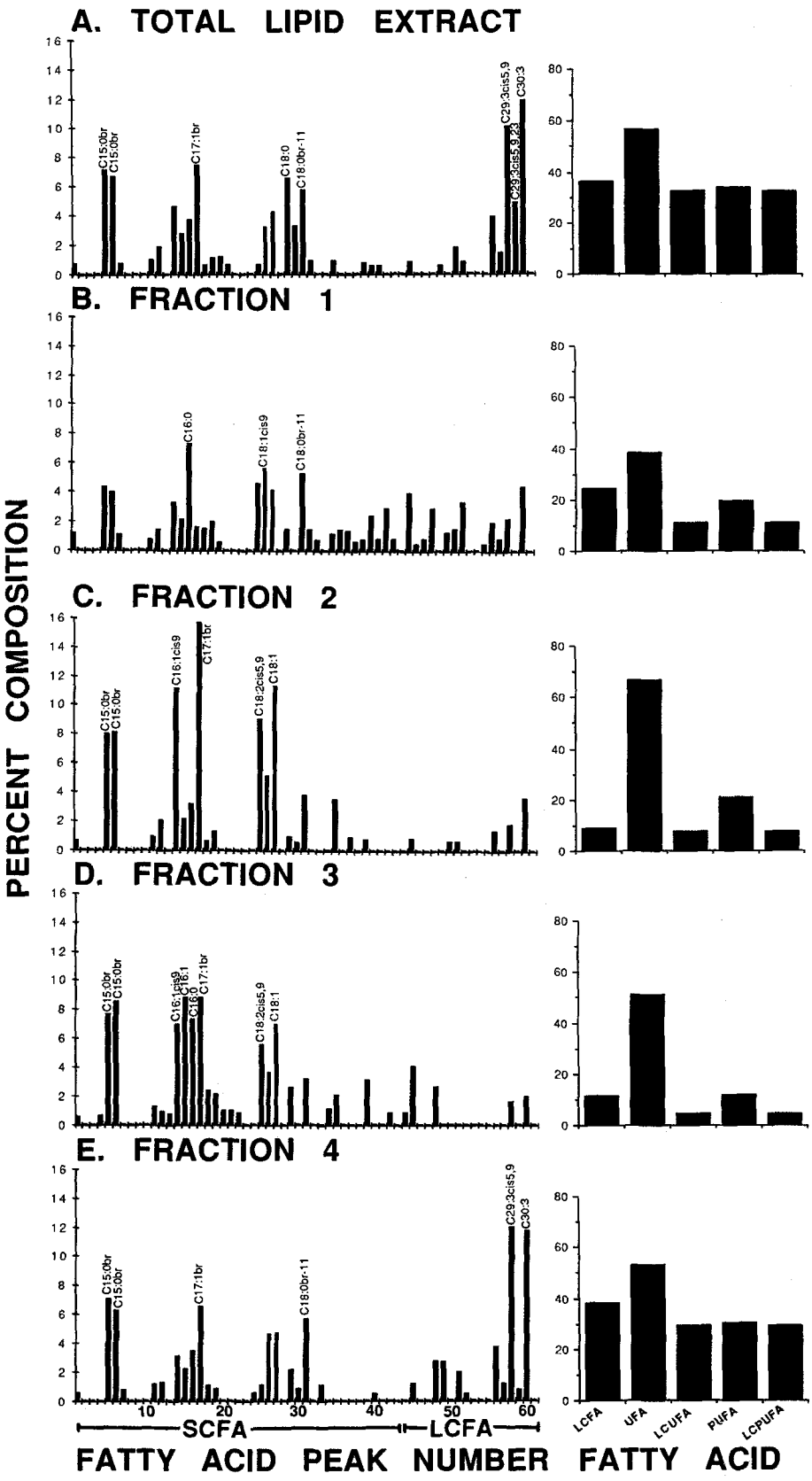

FIG. 2. The distributions of fatty acids as total lipids (A) and in various lipid fractions (B–E) according to fatty acid chain length and saturation for a total lipid extract of *A. fistularis* whole sponge tissue (June collection, Experiment 2). Lipid fractions were expected by convention to contain the following: (B) Fraction 1, triacylglycerols and sterol esters; (C) Fraction 2, free sterols and diacylglycerols; (D) Fraction 3, glycolipids; and (E) Fraction 4, phospholipids. Fatty acids that were ≥5% of the total are labeled; all acids are described in Table 1. SCFA, short chain fatty acids; LCFA, long chain fatty acids (ECL ≥ 23.0); UFA, unsaturated fatty acids; LCUFA, long chain unsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCPUFA, long chain polyunsaturated fatty acids.

than 95% of the acids: C_{15}-C_{18} acids accounted for 63% of the total and C_{28}-C_{30} acids accounted for 32% of the total (Fig. 2). Fatty acids with chain lengths between C_{18} and C_{28} were present in minor amounts (<2% total), suggesting that these acids are either biosynthetic intermediates or dietary acids yet to be processed by the sponge. Most shorter-chain fatty acids in the C_{15}-C_{18} range were found to be branched C_{15}, C_{16} and C_{17} acids, in accordance with previous results for this sponge (5) and for other demosponges (33-37). Fatty acid distribution patterns within each of the two *A. fistularis* collections (March and June) differed (Figs. 2A, 4A) and this is possibly due to seasonal factors, as has been suggested for other sponges (38-40).

Column chromatography of Experiment 1 whole sponge total lipid extract yielded fractions which, according to TLC R_f values, spot characteristics on TLC plates, reference lipids and specific spray reagents, and in accordance with data for other sponges (2,5), were expected to contain the following: Fraction 1—sterol esters (TLC R_f = 0.79 in hexane/ether, 1:1, v/v) and triacylglycerols (0.74); Fraction 2—free sterols and diacylglycerols (0.23); Fraction 3-glycolipids (0.14); and Fraction 4—phospholipids (0). Unidentified lipids were also present at R_f = 0.48.

Of these four lipid fractions, only the phospholipid Fraction 4 contained appreciable levels of long-chain fatty acid (LCFA) (38.4%) (Fig. 2E) and paralleled the levels of LCFA in the total lipid extract (36.3%) (Fig. 2A), although all fractions contained some LCFA (Fig. 2). The major long-chain acids, $C_{28:2cis5,9}$ and $C_{30:3}$, were present at a ratio of 1:1 in the phospholipid fraction, suggesting they co-occur in certain phospholipids or occur singly in phospholipids that complement each other in cell membranes (Fig. 2E). The phospholipid, Fraction 4, was also characterized by high levels of unsaturation (53.3%), especially polyunsaturation in LCFA (Fig. 2E). The similarity of proportions in both LCFA and unsaturated fatty acids between the total lipid extract and the phospholipid, Fraction 4, is common for this (5) and other sponges (1,2), and can be accounted for by the high proportions of phospholipids in sponge tissue (2,5).

Eighteen sterols were detected in the whole sponge total lipid extract described above (Fig. 1). The unconventional sterols 25-dehydroaplysterol (14) and aplysterol (15) accounted for 67.1% of the total sterols, in agreement with the results of Walkup et al. (5), and similar amounts within the sterol esters (64%) and free sterols (65.3%) (Fig. 3). Cholesterol (2) and 5α-cholestan-3β-ol (3) at 13.5-15.3% of the total were the only other sterols present in significant proportions. The sterol composition of free sterols, sterol esters and total sterols was similar (Fig. 3).

Spherulous cell lipid content. The total lipid extract of a subsample of the spherulous cells isolated for Experiment 2 was similar in color to that of the related whole sponge tissue, but differed substantially in lipid composition (Fig. 4A,4B). The spherulous cells lacked several fatty acids that were present in the whole sponge, noteably the $C_{18:0\ br\text{-}11}$ and $C_{18:1}$ (peak no. 28) acids. Moreover, the spherulous cells contained greatly elevated levels of the $C_{16:0}$ acid. It was significant that branched-chain fatty acids were still present in relatively high amounts (12.2%) in spherulous cells, as these acids have been proposed to be bacterial in origin (5).

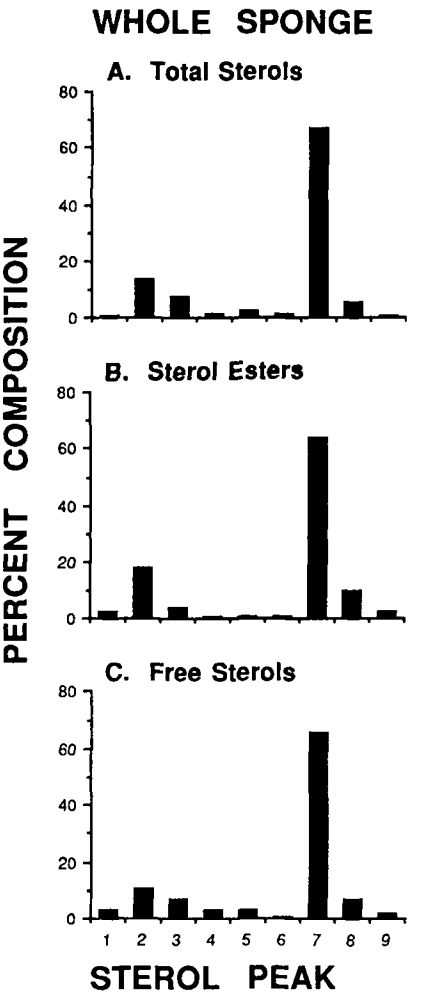

FIG. 3. Relative percent composition of sterols as total sterols (A), sterol esters, (B) and free sterols (C) in *A. fistularis* whole sponge tissue from Experiment 1 (March sample). Sterol peaks: peak 1 = sterol 1; peak 2 = sterols 2 and 3; peak 3 = sterols 4 and 5; peak 4 = sterols 6 and 7; peak 5 = sterols 8-10; peak 6 = sterols 11-13; peak 7 = sterols 14 and 15; peak 8 = sterols 16 and 17; peak 9 = sterol 19. Sterols are identified by number in Figure 1.

Although whole spherulous cell tissue was not analyzed for lipid content for both collections, the P1 pellets, which contained predominantly undisrupted and partially disrupted spherulous cells and organelles, obtained from both subcellular fractionation procedures were analyzed and were found to differ dramatically. The P1 pellet from Experiment 2 contained more than twice as many (ca. 15% more) LCFA, ca. 17% more polyunsaturated fatty acids (PUFA), of which most (~15%) were among LCFA, but lower levels of total unsaturation (~10% less) than the P1 pellet obtained from Experiment 1 (Figs. 4, 5). Also, without exception, the fatty acids that dominated (>5% total) each experimental group were different (Figs. 4C, 5A). Noteably, $C_{22:0}$ occurred at 20.1% in the P1 sample of Experiment 2 spherulous cells, but it was not detected in that of Experiment 1. In contrast, $C_{20:0}$ was present at 8.4% in Experiment 1 P1, but it was not detected in Experiment 2 P1. Long-chain C_{28} to C_{30} acids of Experiment 2 P1 were present in higher proportions (25%) than those of Experiment 1 P1 (10.9%). It was

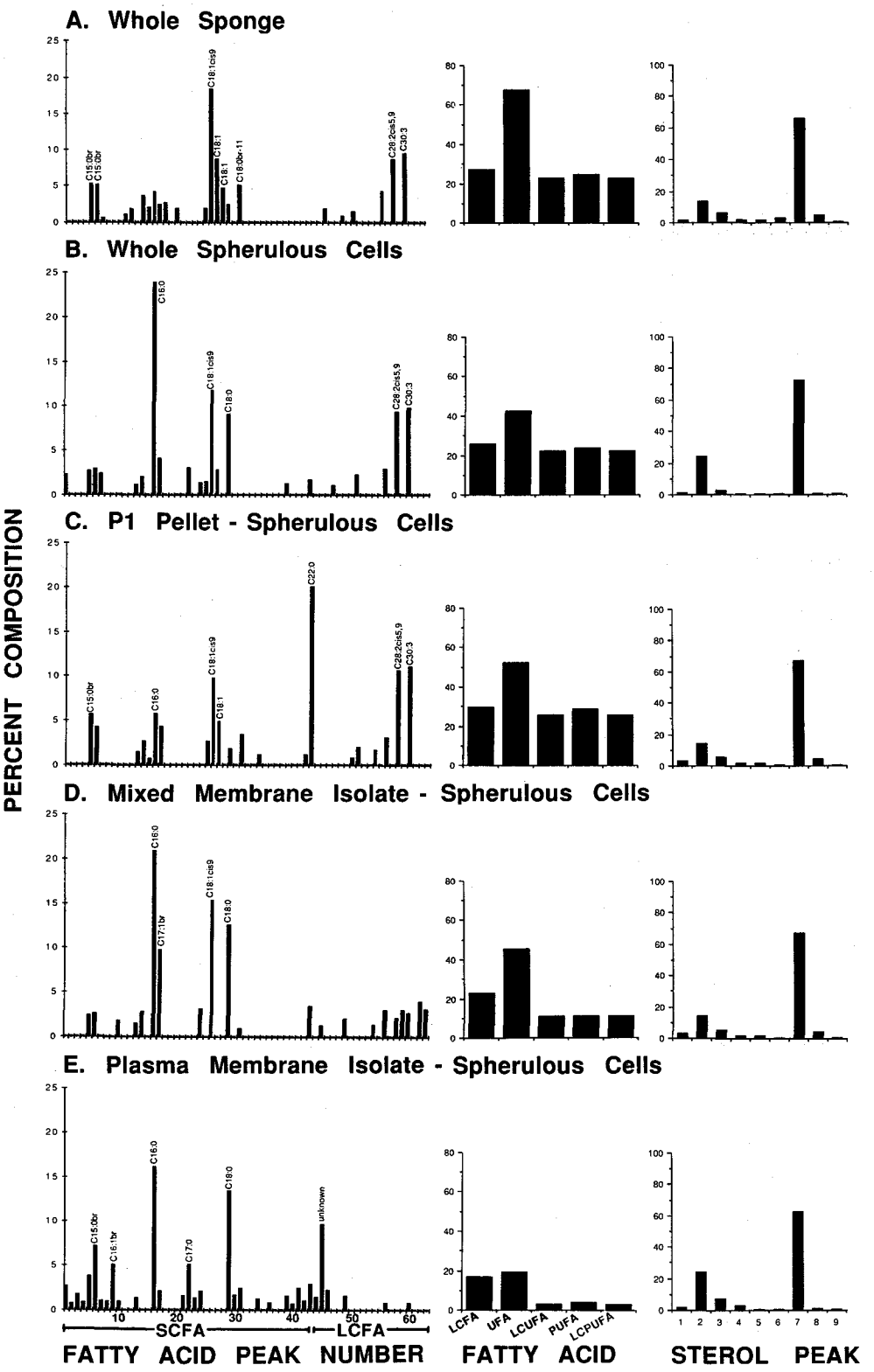

FIG. 4. The distribution of fatty acids, according to fatty acid chain length and saturation, and free sterols in total lipid extracts of whole sponge tissue (A), whole spherulous cells (B), the P1 pellet (C) produced in the course of subcellular fractionation of the spherulous cells, and the two resulting spherulous cell subcellular fractions—i.e., the spherulous cell Mixed Membrane Isolate (D) and the spherulous cell plasma membrane isolate (E), as determined from Experiment 2. Fatty acids that are ≥5% total are labeled; all acids are described in Table 1. Abbreviations and sterol peaks are described in Figures 2 and 3 (captions), respectively.

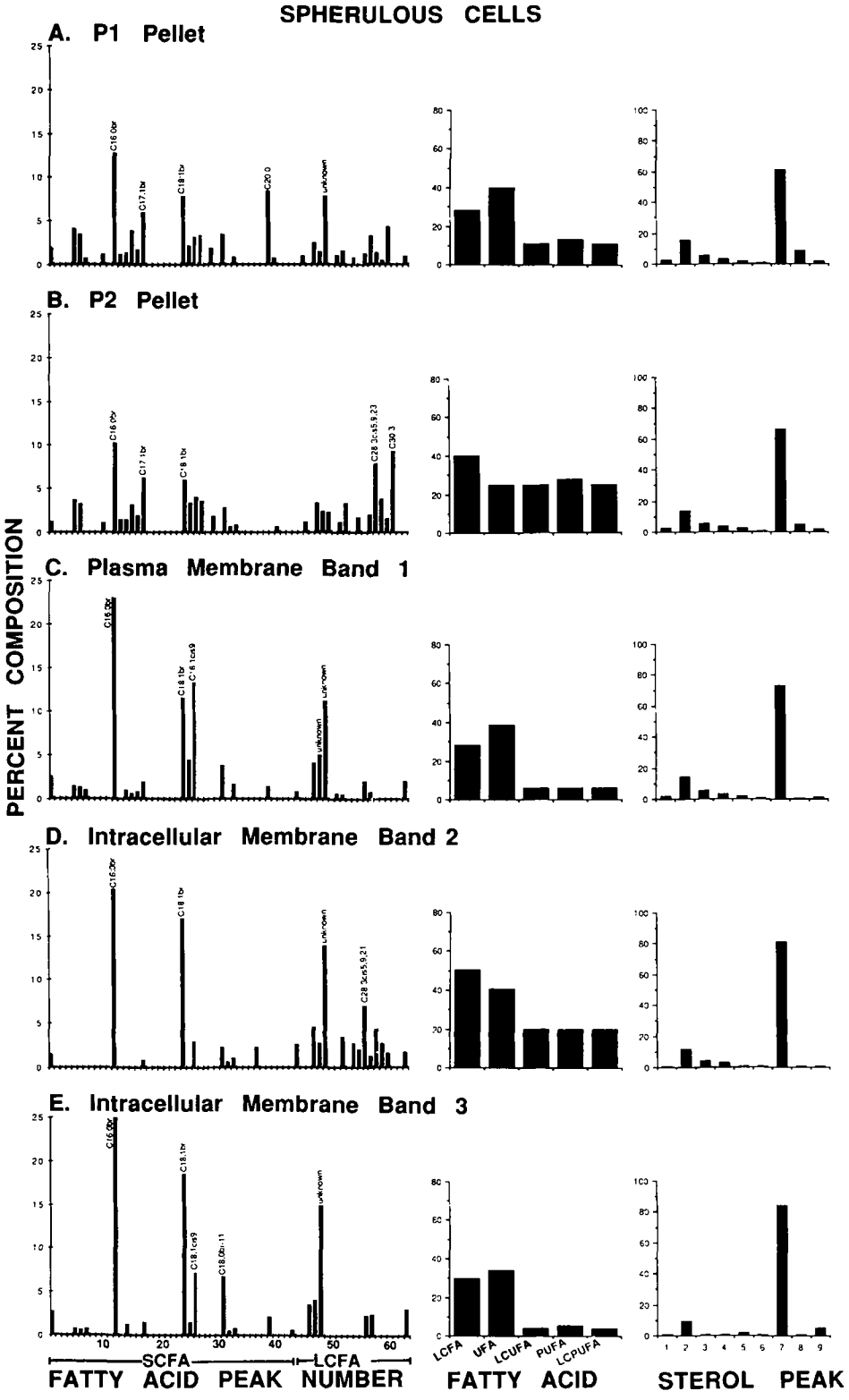

FIG. 5. The distribution of fatty acids, according to fatty acid chain length and saturation, and free sterols in total lipid extracts of spherulous cell subcellular fractions including the P1 (A) and P2 (B) isolates, plasma membrane (C) and intracellular membranes (D and E) as determined from Experiment 1. Fatty acids that are ≥5% total are labeled; all acids are described in Table 1. Abbreviations and sterol peaks are described in Figures 2 and 3 (captions), respectively.

interesting that levels of $C_{28:2cis5,9}$ in Experiment 2 P1 (10.7%) paralleled the level of $C_{30:3}$ (11.2%) in Experiment 2 P1, and $C_{28:3cis5,9,23}$ (3.4%) paralleled the level of $C_{30:3}$ (4.4%) present in Experiment 1 P1. An unknown acid (ECL = 25.479) was present in significant amounts (7.9%) in the P1 sample of Experiment 1, but was absent in that of Experiment 2. Also, the Experiment 1 spherulous cell pellet contained higher levels of branched-chain fatty acids—specifically $C_{16:0br}$, $C_{17:1br}$ and $C_{18:1br}$ acids. Such differences in lipid composition are possibly due to seasonal factors, as mentioned for whole sponge tissue.

Similarities in the fatty acid composition were observed between the P2 pellet (Fig. 5), which was found to consist largely of spherules, and that of Intracellular Membrane Band 2 in Experiment 1 (Fig. 5D) suggesting that Intracellular Membrane Band 2 represents spherule membranes. For example, Intracellular Membrane Band 2 of Experiment 1 had high LCFA (48.1%) and PUFA (19.7%) levels which were reminiscent of the P2 'spherule' pellet where LCFA and PUFA totaled 39.8% and 27.8%, respectively.

The principal phospholipids of the spherulous cells were phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine and phosphatidylinositol, with a minor content of phosphatidylglycerol and diphosphatidylglycerol, in accordance with data for whole tissue (5).

All sterols detected in the whole sponge were also detected in the related spherulous cells, but the relative proportions of the sterols were different (Figs. 3, 4B). 25-Dehydroaplysterol (14) and aplysterol (15) were enriched in the spherulous cells (72.2%), as compared with whole sponge tissue (67.1%); and cholesterol (2) and 5α-cholestan-3β-ol (3) amounted to 24.2% of the total in spherulous cells and 13.8% in the whole sponge. The sterol composition of the P1 samples from both Experiments were similar except that there was slightly more (24S[or R])-isopropropenylcholesta-5,22-dien-3β-ol (16) and 7-dehydroaplysterol (17) (peak 8, Fig. 5A) in the P1 isolate of Experiment 1 than in that of Experiment 2.

Membrane isolate lipid content. Membrane lipids were transparent and mainly consisted of phospholipid and sterol, but sterol esters and an unidentified lipid (R_f = 0.48 in hexane/ether, 1:1) also were present in lesser amounts. It was not determined whether these lipids were situated totally or partially within the membrane bilayer, or whether they were peripheral molecules adherent to the exterior/interior membrane surface(s).

The spherulous cell plasma membrane isolates produced by both experimental methods contained appreciable levels of LCFA: 27.2% of the fatty acids from the Experiment 1 Plasma Membrane Band 1 and 16.8% of the fatty acids from the Experiment 2 Plasma Membrane Isolate were LCFA. For Experiment 1, this plasma membrane LCFA content (27.2%) mirrored that observed for the corresponding P1 pellet (28.2%, Fig. 5), but for Experiment 2, this LCFA content (16.8%) was significantly below that observed for the corresponding P1 pellet (29.5%) or whole tissue (27.3%) (Fig. 4). Spherulous cell plasma membrane LCFA were predominantly of shorter chain lengths (with the measured ECL equaling 24.75-25.48 [$\sim C_{25}$, peak nos. 44-49; 21.21%—Experiment 1; 15.1%—Experiment 2]) than were found in whole spherulous cells or in intracellular membranes (Figs. 4,5), and longer LCFA (peak nos. 50-63, chain lengths C_{26}-?C_{33}) occurred in comparatively low levels (6.8%—Experiment 1; 1.7%—Experiment 2).

The intracellular membrane isolates also contained appreciable levels of LCFA, but the relative fatty acid compositions differed greatly, suggesting different intracellular membranes may differ substantially in composition. One of the Experiment 1 Intracellular Membrane Isolates contained a higher level of LCFA than did the corresponding Plasma Membrane Isolate: Intracellular Membrane Band 2 contained 48.1% LCFA (Fig. 5D), and the corresponding Plasma Membrane Band 1 contained only 27.2% LCFA (Fig. 5C). The Experiment 2 Mixed Membrane Isolate of Percoll gradient 1, which contained a mixture of intracellular membranes and plasma membrane of which the proportions are unknown, contained 22.8% LCFA (Fig. 4D), as compared with 16.8% in the plasma membrane (Fig. 4E). The Experiment 1 Intracellular Membrane Band 3, however, contained 29.8% LCFA—approximately the same as the corresponding Plasma Membrane Band 1 (Fig. 5). Also, Intracellular Membrane Band 3 isolate contained principally shorter LCFA (ECL = 24.75-24.84; C_{24}-C_{25}), and the Intracellular Membrane Band 2 contained more longer acids (ECL = 24.75-28.68; C_{25}-C_{29}). The Experiment 2 Mixed Membrane Isolate likewise contained longer range acids (ECL = 27.49-32.70). The spherulous cell plasma membrane isolate of Experiment 2 had a lower LCFA (16.8%), unsaturated (19.1%) and polyunsaturated (4.1%) fatty acid content and different individual fatty acid composition to that of the parent Mixed Membrane Isolate where LCFA, unsaturated and polyunsaturated fatty acids amounted to 21.5%, 35.5% and 10.9%, respectively.

Further differences in the fatty acid composition between plasma membrane isolates and their corresponding P1 pellets were observed. For example, in Experiment 1, the $C_{18:1cis9}$ acid was greatly enriched in the Plasma Membrane Band 1 although it was barely detectable in the related spherulous cell P1 pellet. Also, P1 had a higher content of the longer LCFA (peak nos. 50-63, chain lengths C_{26}-C_{33}) at 29.5% for Experiment 2 P1 and 15.3% for Experiment 1 P1, although not necessarily of total LCFA, which amounted to 29.5% for Experiment 2 P1 and 28.2% for Experiment 1 P1, compared with a total LCFA content of 27.2% for the Experiment 1 plasma membrane isolate and only 3.4% for the Experiment 2 plasma membrane isolate.

It is not expected that spherulous cell plasma membrane reflects the fatty acid composition of parent spherulous cells (or the P1 pellet) because: 1) other subcellular membranes within spherulous cells (e.g., spherule boundary membranes with high surface areas) are expected to contribute more than plasma membrane to the total fatty acid pool and 2) fatty acids may be stored in subcellular organelles within spherulous cells.

Plasma membrane isolates from both experiments exhibited variable levels of unsaturation (38.5% in Experiment 1; 19.1% in Experiment 2) (Figs. 5C, 4E). Intracellular Membrane Bands 2 and 3 of Experiment 1 exhibited high levels of unsaturation at 40.4% and 34.1%, respectively (Fig. 5D, 5E). The Experiment 2 plasma membrane isolate also exhibited a lower level of LCFA unsaturation (16.8%), relative to the Mixed Membrane Isolate (22.8%) (Fig. 4). In all cases, virtually all polyunsaturation occurred in the LCFA (Figs. 4, 5).

Spherulous cell plasma membrane from each experiment also exhibited major differences in the composition of individual fatty acids (Figs. 4C, 4E, 5A, 5C). Again, this may be due to environmentally or seasonally induced differences in fatty acid content.

All sterols detected in whole sponge tissue were also detected in the spherulous cell plasma and intracellular membranes, and in the P1 and P2 isolates (Figs. 4, 5), although there were some differences in the relative contributions of each sterol in these samples. All samples were dominated by cholesterol (2) and 5α-cholestan-3β-ol (3) (peak no. 2) and 25-dehydroaplysterol (14) and aplysterol (15) (peak no. 7), which represented 9.4–24.2% and 60.9–80.8%, respectively. Spherulous cell plasma membrane contained 72.6% (Experiment 1) and 66.4% (Experiment 2) of the unconventional sterols 25-dehydroaplysterol (14) and aplysterol (15) (Figs 5C, 4E). For Experiment 1 spherulous cell plasma membrane, this was ca. 10% less than that found in the intracellular membranes, although no such difference could be observed between the plasma membrane and Mixed Membrane Isolate of Experiment 2 (Figs. 4, 5), supporting our earlier claim that the Mixed Membrane Isolate was largely plasma membrane. Differences in the proportions of minor sterols between subcellular fractions also were observed. (24S[or R])-Isopropropenylcholesta-5,22-dien-3β-ol (16) and 7-dehydroaplysterol (17) (peak no. 8) occurred at 8.6% in P1, but about half this amount in P2 (4.8%) and at only trace levels (<1%) in all membrane isolates of Experiment 1 (Fig. 5). In Experiment 2, sterols 16 and 17 were present at about twice the amount in P1 (4.1%) as in the Mixed Membrane (1.3%) and plasma membrane isolates (1.4%), although only half that of Experiment 1 P1 (Fig. 4). Crinosterol (4) and brassicasterol (5) (peak 3) were present in trace amounts in Intracellular Membrane Band 3, compared with higher levels (4.3–5.8%) in all other subcellular samples of Experiment 1 (Fig. 5). In Experiment 2, these sterols occurred at 7.7% in the Mixed Membrane Isolate, but in lower proportions in the plasma membrane (2%) and P1 pellet (5.8%) (Fig. 4). E/Z-Cholesta-5,22-dien-3β-ol (1) (peak no. 1) was observed in lower levels in Intracellular Membrane Bands 2 and 3 (trace amounts) than in plasma membrane (1.8%) of Experiment 1 (Fig. 5). Similarly, in Experiment 2, the Mixed Membrane Isolate contained lower levels of this sterol (1.9%) compared with the plasma membrane (3.7%) and P1 pellet (3.3%) (Fig. 4). Verongulasterol (18) (peak 9) occurred at 4.9% in Intracellular Membrane Band 3, but at less than half this in the plasma membrane and the remaining subcellular isolates in Experiment 1 (Fig. 5). Differences in the proportions of sterols for the two experiments may be attributable to seasonal factors, such as environmental temperature or the sponge diet, but the significance of these differences is unknown.

Sterol:phospholipid:protein ratios of subcellular isolates. Fractions identified as spherulous cell plasma membrane by other methods were found to possess greater sterol:phospholipid and sterol:protein ratios than other subcellular membrane systems isolated in this study (Table 2), in concordance with sterol:phospholipid and sterol:protein ratios reported for other plasma membranes (18).

DISCUSSION

This study presents the first definitive evidence for the presence of unconventional lipids in a subcellular membrane system of a marine sponge and thereby supports earlier proposals that unconventional sponge lipids are membrane constituents (1–3,5,33,39,41–44). Conventional subcellular fractionation methods were successfully used to produce highly pure plasma membrane from *A. fistularis* spherulous cells; the purity of these plasma membrane isolates was established by: (1) the enrichment of a radioactive plasma membrane marker, (2) the presence and absence of specific polypeptides and (3) the existence of characteristic protein:sterol:phospholipid ratios. Long-chain fatty acids (LCFA) and the unique sponge sterols, aplysterol (15) and 25-dehydroaplysterol (14), were confirmed as major components of spherulous cell plasma membrane in *A. fistularis*, in contrast to the short-chain (C_{14}–C_{22}) fatty acids (SCFA) and comparatively simple sterol molecules that are exclusively present in the cell membranes of most living organisms.

This study also documents that more conventional lipids co-occur with the unconventional lipids in the *A. fistularis* spherulous cell plasma membrane. The unconventional sterols aplysterol (15) and 25-dehydroaplysterol (14) were found to constitute 66.4–72.6% of the sterols, but the bulk of the remaining sterols were the conventional sterols cholesterol (2) and 5α-cholestan-3β-ol (3). LCFA were found to only constitute 16.8–28.0% of the total fatty acids; the remainder of the fatty acids were C_{14}–C_{22} lengths, including substantial quantities of branched C_{15}–C_{18} fatty acids, which are typical of bacterial cell membranes, but not of animal membranes. This does not necessarily mean that branched acids are synthesized de novo by the sponge. Given our own biosynthetic studies (37,45), short-chain (<C_{22}) branched acids are obtained either through the diet or from the abundant

TABLE 2

The Sterol:Phospholipid:Protein Ratios (mg/mg) of Spherulous Cells, Plasma Membrane and Subcellular Isolates From *A. fisturalis*

	Plasma membrane		Subcellular membranes			Experiment 1 Spherulous cells	
	Experiment 1	Experiment 2	Experiment 1 Intracellular Membrane Band 2	Experiment 1 Intracellular Membrane Band 3	Experiment 2 Mixed Membrane Isolate	Whole	P1 pellet
Sterol:Phospholipid	0.491	0.514	0.131	0.118	0.445	0.296	0.163
Sterol:Protein	0.327	0.392	0.104	0.159	0.300	0.064	0.096

symbiotic bacteria present in the sponge tissue, and their long-chain counterparts are synthesized by the sponge by chain elongation and desaturation of the shorter precursors.

Further investigations are required to establish the lipid contents of the intracellular membranes present within *A. fistularis* spherulous cells, but examination of undefined intracellular membrane isolates produced from spherulous cells during the course of this study indicates that there is considerable variability among the various types of membranes in this sponge. For example, although spherulous cell plasma membrane contained appreciable quantities of LCFA, the highest observed quantities of LCFA occurred in one unidentified intracellular membrane isolate.

Although it is certain that the plasma membrane lipid composition documented for *A. fistularis* spherulous cells does not reflect that of all sponge plasma membranes, as there is considerable variability in the lipid contents of different sponges (not all sponges contain unconventional sterols [46] and some species contain very low levels of LCFA [47]), it is not known if plasma membrane composition varies between cell types in *A. fistularis*. The fact that isolated spherulous cells differ from the corresponding whole tissue, however, suggests there may be variation according to cell type within this sponge. Moreover, it is uncertain whether plasma membrane composition varies between individual spherulous cells (e.g., according to size or age).

Further chemical characterization of additional sponge cell membranes is required to answer basic questions concerning the structures and functions of these membrane systems. Progress will be most quickly made if membrane isolates can be prepared from single cell types that are of a similar life history stage. Ideally, this might be achieved through in vitro culturing of particular sponge cell types, but so far this has proven impossible to achieve because of sensitivity of sponge cells to alien environments and their susceptibility to differention into varying cell types.

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REFERENCES

1. Lawson, M.P., Bergquist, P.R., and Cambie, R.C. (1986) *Tissue Cell 18,* 19-26.
2. Lawson, M.P., Thompson, J.E., and Djerassi, C. (1988) *Lipids 23,* 741-749.
3. Lawson, M.P., Stoilov, I.L., Thompson, J.E., and Djerassi, C. (1988) *Lipids 23,* 750-754.
4. De Rosa, M., Minale, L., and Sodano, G. (1973) *Comp. Biochem. Physiol. 45B,* 883-893.
5. Walkup, R.D., Jamieson, G.C., Ratcliff, M.R., and Djerassi, C. (1981) *Lipids 16,* 631-646.
6. Catalan, C.A.N., Thompson, J.E., Kokke, W.C.M.C., and Djerassi, C. (1985) *Tetrahedron 41,* 1073-1084.
7. Thompson, J.E., Barrow, K.D., and Faulkner, D.J. (1983) *Acta Zool. 64,* 199-210.
8. Vacelet, J. (1967) *J. Microsc. 6,* 237-240.
9. Bretting, H., Günter, J., Donadey, C., and Vacelet, J. (1982) in *Lectins-Biology, Biochemistry, Clinical Biochemistry* (Bog-Hansen, T.C., ed.) Vol. II, pp. 91-103, W. de Gruyter & Co., Berlin, East Germany.
10. Thompson, J.E. (1984) *Mar. Biol. 88,* 23-26.
11. Bretting, H., and Königsmann, K. (1979) *Cell Tissue Res. 201,* 487-497.
12. Fauvel, J., Chap, H., Roques, V., Levy-Toledano, S., and Douste-Blazy, L. (1986) *Biochim. Biophys. Acta 856,* 155-164.
13. Sorimachi, K. (1984) *Biochem. Biophys. Res. Commun. 125,* 35-44.
14. Cuatrecasas, P. (1973) *Biochemistry 12,* 1312-1323.
15. Juliano, R.L., and Li, G. (1978) *Biochemistry 17,* 678-683.
16. Emerson, D., and Juliano, R.L. (1982) *J. Cell Physiol. 111,* 171-176.
17. Chicken, C.A., and Sharom, F.J. (1985) *Can. J. Biochem. Cell Biol. 63,* 64-70.
18. Graham, J.M. (1975) in *New Techniques in Biophysics and Cell Biology* (Pain, R.H., and Smith, B.J., eds.) Vol. 2, pp. 1-43, John Wiley & Sons, London, England.
19. Record, M., Bes, J-C., Chap, H., and Douste-Blazy, L. (1982) *Biochim. Biophys. Acta 668,* 57-65.
20. Scharschmidt, B.F., Keeffe, E.B., Blankenship, N.M., and Ockner, R.K. (1979) *J. Lab. Clin. Med. 93,* 790-797.
21. Weber, K., and Osborn, M. (1969) *J. Biol. Chem. 244,* 4406-4412.
22. Laemmli, U.K. (1970) *Nature 277,* 689-685.
23. Ames, G.F.L. (1979) *J. Biol. Chem. 249,* 634-644.
24. Andersson, B.Å. (1978) *Prog. Chem. Fats Other Lipids 16,* 279-308.
25. Heider, J.G., and Boyett, R.L. (1978) *J. Lipid Res. 19,* 514-518.
26. Peterson, G.L. (1977) *Anal. Biochem. 83,* 346-356.
27. Johnson, S.M. (1979) *Anal. Biochem. 95,* 344-350.
28. Bartlett, G.R. (1959) *J. Biol. Chem. 234,* 466-468.
29. Bosmann, H.B., Hagopian, A., and Eylar, E.H. (1968) *Arch. Biochem. Biophys. 128,* 51-69.
30. Gorshkov, B.A., Gorshkova, I.A., Makarieva, T.N., and Stonik, V.A. (1982) *Toxicon 20,* 1092-1094.
31. Gorshkov, B.A., Gorshkova, I.A., and Makarieva, T.N. (1984) *Toxicon 22,* 441-449.
32. Aronson, N.N. Jr., and Touster, O. (1974) in *Methods in Enzymology* (Fleisher, A.S., and Packer, L., eds.) Vol. XXXI, pp. 91-102, Academic Press, New York, NY.
33. Ayanoglu, E., Walkup, R.D., Sica, D., and Djerassi, C. (1982) *Lipids 17,* 617-625.
34. Lankelma, J., Ayanoglu, E., and Djerassi, C. (1983) *Lipids 18,* 853-858.
35. Wijekoon, W.M.D., Ayanoglu, E., and Djerassi, C. (1984) *Tetrahedron Lett. 25,* 3285-3288.
36. Dasgupta, A., Ayanoglu, E., and Djerassi, C. (1984) *Lipids 19,* 768-776.
37. Carballeira, N., Thompson, J.E., Ayanoglu, E., and Djerassi, C. (1986) *J. Org. Chem. 51,* 2751-2756.
38. Jefferts, E., Morales, R.W., and Litchfield, C. (1974) *Lipids 9,* 244-247.
39. Litchfield, C., and Morales, R.W. (1976) in *Aspects of Sponge Biology* (Harrison, F.W., and Cowden, R.R., eds.) pp. 183-200, Academic Press, New York, NY.
40. Lawson, M.P. (1984) Sponge Lipids, Ph.D. Thesis, University of Auckland, New Zealand, 185 pp.
41. Djerassi, C., Theobold, N., Kokke, W.C.M.C., Pak, C.S., and Carlson, M.K. (1979) *Pure Appl. Chem. 51,* 1815-1828.
42. Carlson, R.M.K., Tarchini, C., and Djerassi, C. (1980) in *Frontiers of Bioorganic Chemistry and Molecular Biology* (Ananchenko, S.N., ed.) pp. 211-224, Pergamon Press, New York.
43. Djerassi, C. (1981) *Pure Appl. Chem. 53,* 873-890.
44. Bergquist, P.R., Lawson, M.P., Lavis, A., and Cambie, R.C. (1984) *Biochem. Syst. Ecol. 12,* 375-393.
45. Raederstorff, D., Shu, A.Y.L., Thompson, J.E., and Djerassi, C. (1986) *J. Org. Chem. 52,* 2337-2756.
46. Bergquist, P.R., Hofheinz, W., and Oesterhelt, G. (1980) *Biochem. Syst. Ecol. 12,* 63-84.
47. Lawson, M.P., Bergquist, P.R., and Cambie, R.C. (1984) *Biochem. Syst. Ecol. 12,* 375-393.

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Fatty Acid Composition of Fats of Differing Melting Points Extracted From Ram Subcutaneous Tissue

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The observation that the subcutaneous fat of pasture-fed Southdown rams consists of two distinct regions is reported. Fatty acid composition of fat from the outer and inner regions of subcutaneous tissue taken from the rib region of eight Southdown rams fed pasture were determined. Relative to the harder inner regions (mean melting point 43.1°C), the softer outer regions (mean melting point 31.8°C) were shown to contain more 9:0-, 15:0-, 17:0-, 17:1-, 18:1-*cis* and total 18:1 fatty acids; less 14:0-, 16:0-, 18:0- and 18:1-*trans* fatty acids; and a greater variety and a greater concentration of branched-chain components. Proportions of medium chain-length fatty acids, other than 9:0, did not differ between the layers.

The fatty acid contents of serial samples taken at 1-mm intervals through these tissues were determined. Changes in concentrations of components among samples were gradual through the tissues. There was no clear connective tissue sheet, as has been reported for pigs. The inner region of the tissues contains apparently nonrandom fluctuating changes in fatty acid composition.

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The composition of ovine subcutaneous fat may be influenced by what the animals are fed (1). Diets containing high levels of cereals fed to ewes and wethers may result in the animals having softer subcutaneous fats compared to those fed pasture (2). Subcutaneous fats from the cereal-fed animals may contain higher concentrations of saturated branched-chain, n-saturated odd-carbon and monoenoic acids, and lower concentrations of n-saturated even-carbon chain acids than the corresponding fats from pasture-fed animals (2–6). Similar dietary effects on the fats from rams have also been reported (7,8).

One of us (RWP) observed that the subcutaneous fat of pasture-fed Southdown rams consisted of two distinct regions, the outer of which was very soft—almost oily. Among the animals studied, the soft region varied in thickness from being barely discernible to about 5-mm thick. Investigations of this phenomenon have not been previously reported. To ascertain the chemical reason for their differences, the fatty acid compositions of these regions were determined. Both medium and long chain-length acids were analyzed. Results of these analyses are reported here.

MATERIALS AND METHODS

A group of eight Southdown rams that had been fed on pasture (swards containing ca. equal amounts of white clover and perennial ryegrass) for about 18 months from weaning were slaughtered and the two distinct regions of subcutaneous tissue were separated from an area over ribs 8–12. Samples from the two regions were extracted with chloroform and the solvent then removed under reduced pressure. The extracts accounted for ca. 90% of the original tissues. They were shown by thin layer chromatography (9) to be principally triglycerides. Melting points of the fats were determined by AOCS Method Cc 1-25 (10).

Acylglycerols were converted into methyl esters by treatment with methanolic KOH followed by BF_3/methanol (11). Saturated long chain-length methyl esters were separated from unsaturated esters by adsorption of mercuric acetate adducts of the latter compounds on Amberlite CG-50 type 1 weak cation exchange resin (Johnson, C.B., unpublished data). Esters thus fractionated, as well as the original mixtures, were analyzed by gas chromatography using a Hewlett-Packard Model 5840 chromatograph. Unsaturated esters were separated on a 7 m × 2.3 mm i.d. stainless steel column containing silicone OV-275 (15% on Chromosorb P) at 220°C with a carrier gas (nitrogen) flow rate of 10 ml/min. The original mixtures of esters and the saturated esters were separated on a 2 m × 2.3 mm i.d. stainless steel column containing EGSS-X (10% on Chromosorb W) at 180°C with a carrier gas flow rate of 30 ml/min. A similar column containing Apiezon L grease (3%) at 200°C was also used for the separation of saturated esters.

Medium chain-length acids in the fats were isolated by steam distillation, esterified and analyzed on a 3.7 m × 2.3 mm i.d. stainless steel column containing 10% silicone OV-101 on Gas Chrom Q, as previously reported (12).

Cube samples (ca. 10 mm^3) of the intact adipose tissues from 2 animals were placed on aluminum discs and cooled to −15°C. The discs were placed in a cooled microtome and slices 1-mm thick were then cut sequentially parallel to the outer surfaces from the mounted samples. In addition, from two more animals, the hard and soft regions from the two samples were separated and sliced as described. Fat extracted from the slices was analyzed as described above.

RESULTS

The composition of the fatty acids from the inner and the outer regions of subcutaneous fat from the eight Southdown rams is given in Table 1. Also presented are the melting points of the fats. Fats from the outer regions melted at temperatures between 25.1°C and 39.8°C (mean 31.8°C) and, thus, felt oily. The inner regions were hard, having melting points between 41.4°C and 45.1°C (mean 43.1°C), similar to that of normal ewe or wether fats (13).

Most outer fats contained more 4-methyloctanoic acid and 9:0 than the inner fats. These levels were much higher than those found previously and, thus, may have implications for flavor of the cooked meat (12).

Relative to the inner regions of fat, the outer regions had a significantly greater quantity of n-saturated acids containing odd numbers of carbon atoms and a lesser quantity of the corresponding acids with even numbers of carbon atoms. The outer regions also contained a

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TABLE 1

Melting Points (Means) and Fatty Acid Composition (Mean) of Fats Extracted From the Inner and Outer Regions of Subcutaneous Tissue From Southdown Rams

	Fat layer		Difference (O − I)		
	Outer (O)	Inner (I)	Mean	SE[a]	Significance[b]
M.P.[c]	31.8	43.1	−11.3	1.5	***
Medium chain-length acids (ppm)					
6:0[d]	109	98	12	29	NS
7:0	175	112	63	42	NS
8:0	258	219	39	54	NS
9:0 (br)[e]	204	116	88	41	NS
9:0	277	177	100	21	**
Long chain-length acids (%)					
10:0	0.09	0.13	−0.03	0.01	***
u[f]	0.11	0.03	0.08	0.01	***
11:0	0.05	0.01	0.04	0.01	***
u	0.20	0.03	0.17	0.02	***
u	0.22	0.01	0.21	0.02	***
12:0	0.00	0.08	−0.08	0.01	***
u	0.27	0.08	0.19	0.02	***
u	0.21	0.04	0.17	0.03	***
13:0	0.12	0.02	0.10	0.03	**
u	0.28	0.03	0.25	0.03	***
u	0.56	0.10	0.46	0.09	***
14:0	1.82	2.51	−0.69	0.10	***
14:1 (c)	0.19	0.25	−0.06	0.04	NS
14:1 (t)	0.07	0.03	0.04	0.01	***
15:0 (i)	0.07	0.17	−0.10	0.04	NS
15:0 (ai)	0.40	0.31	0.09	0.04	NS
15:0 (br)	2.16	0.63	1.53	0.10	***
15:0	1.62	0.88	0.74	0.08	***
16:0 (i)	2.70	0.53	2.17	0.28	***
16:0	15.4	19.5	−4.1	0.4	***
16:1 (c)	1.61	1.36	0.25	0.13	NS
16:1 (t)	0.17	0.42	−0.26	0.04	***
17:0 (i)	0.28	0.35	−0.07	0.09	NS
17:0 (ai)	1.58	0.86	0.72	0.13	***
17:0 (br)	3.55	0.82	2.73	0.39	***
17:0	3.93	2.42	1.51	0.37	**
17:1 (c)	2.71	0.78	1.93	0.30	***
17:1 (t)	0.10	0.05	0.05	0.02	NS
18:0 (i)	0.97	0.28	0.69	0.09	***
18:0	11.0	24.3	−13.2	0.7	***
18:1 (c)	37.0	29.2	7.8	1.2	***
18:1 (t)	4.68	7.95	−3.27	0.70	***
18:2 (c,t)	0.42	1.31	−0.89	0.09	***
18:2 (c,c)	2.26	2.61	−0.35	0.23	NS
18:3 (c,c,c)	2.39	1.96	0.43	0.28	NS
19:1	0.37	0.13	0.24	0.11	NS

[a]Standard error of the means.

[b]Significance of the outer/inner difference, based on a paired t-test (n = 8).

[c]Melting point (°C).

[d]Chain-length:number of double bonds. c = *cis*, t = *trans*, i = iso, ai = anteiso, br = branched other than iso or anteiso.

[e]4-Methyloctanoic acid (hircinoic acid).

[f]Of unknown structure, probably branched saturated (see text).

NS, not significant, $P > 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

greater proportion of anteiso and other branched-chain acids (labeled br in Table 1 [5,14]). Overall, there were significantly more branched-chain acids in the outer, than in the inner fats.

A noticeable feature of the gas chromatograms of methyl esters derived from the outer fats was the large number of small peaks (most <0.5%) between the solvent peak and that of 14:0. These peaks did not shift when the methyl esters were treated with hydrogen and Adam's catalyst (13). Thus, most of them probably were saturated branched-chain compounds. The total concentrations of these compounds were in the ranges 0.96–3.23% (mean 1.87%) in the outer fats and 0.13–0.65% (mean 0.32%) in the inner fats. Position location of the branches and determination of their chain-lengths were not undertaken.

The proportions of the monoenoics 17:1-*cis* and 18:1-*cis* were greater in the outer fats. Inner fats contained a greater proportion of 18:1-*trans* compared with the outer fats. *Cis*-polyunsaturated acids did not differ between the layers, but there was a consistently higher level of 18:2-*cis,trans* isomers in the inner fats.

Slides prepared from the subcutaneous fat tissue that had two clear regions differing in softness and color did not reveal a clear connective tissue sheet separating the layers, as has been reported for pigs (15). The connective tissue present (as shown by van Gieson's stain [16]) was spread throughout the subcutaneous tissue, forming numerous layers and lobules.

Analyses of 1-mm samples taken sequentially through the subcutaneous tissue showed that the fats in the two regions of differing hardness were not homogeneous mixtures of acylglycerols. The concentrations of branched-chain, *cis*-unsaturated and n-saturated components containing an odd number of carbon atoms decreased across the soft region from the outer slice. Concentrations of these components also tended to decrease across the harder inner region. However, the apparent nonrandom distribution of points on either side of a quadratic regression line for each component indicate that there were regular fluctuations in composition of the fat across the inner region. This is illustrated in Figure 1 (for a single animal) for n-heptadecanoate and the methyl branched-chain pentadecanoate (other than the iso and anteiso isomers) designated br-15:0. Concentrations of n-saturated components containing an even number of carbon atoms and *trans*-unsaturated components increased throughout the tissues from the outside to the inside. In most instances, the increase across the hard inner region was less than that across the soft outer region. However, for some samples, the increase in concentration of n-octadecanoate (Fig. 1) was almost linear throughout the subcutaneous tissue.

DISCUSSION

The subcutaneous fats of sheep are more unsaturated and of lower melting point than their perinephric and other internal depot fats (17). Differences in melting points among these fats may also be attributed to differences in the proportions of palmitate and stearate (18). Gradients of decreasing levels of unsaturated acids in the subcutaneous fat between the skin and muscle tissue have been observed in cattle, sheep and goats (19), as well as in pigs (15). However, pig subcutaneous fat consists of two layers of different composition on either side of an interface (15,20). The current paper records the first observation and analysis of two regions of different softness (or oiliness) in pasture-grazed ovine ram fats.

Melting points of the fats from the two layers are controlled principally by the levels of their main components,

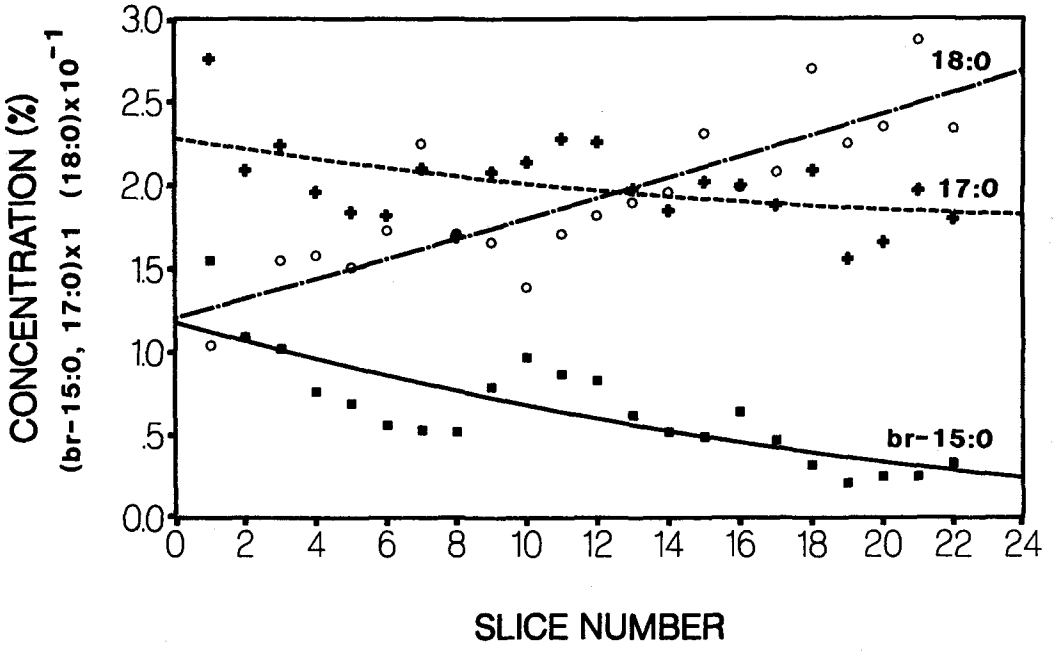

FIG. 1. Changes in fatty acid concentrations with distance into a subcutaneous tissue from surface (slice). Data for each 1-mm slice are shown together with quadratic regression lines for 18:0 (○, —·—·—), 17:0 (+, – – – –) and br-15:0 (■, ——). The visually soft region was 4-mm thick.

namely the 16:0-, 18:0- and 18:1-*cis* and *trans* moieties. These constituted 59–74% (mean 68%) of the outer fat and 79–84% (mean 81%) of the inner fat in the eight animals studied. Decreased melting points of the fats were associated with lower proportions of 16:0-, 18:0- and 18:1-*trans* and higher proportions of 18:1-*cis*.

Other acylglycerol moieties are individually present in small amounts (0.01–5.0%) and, thus, would have little effect on the melting point of the resulting fat. However, their cumulative effect could be important.

Fatty acids and esters containing an odd number of carbon atoms have lower melting points than the corresponding compounds having one less methylene group in their chain (21,22). Likewise, *cis*-unsaturated and branched-chain saturated acids have lower melting points than the corresponding *trans*-unsaturated and n-acids, respectively. Enhanced amounts of 15:0, 17:0, branched-chain saturated and *cis*-unsaturated moieties in the outer fats compared with the inner fats is consistent with the lower melting points of these fats.

Triacylglycerols from the outer layer of pig subcutaneous fat contain more unsaturated acids, less saturated acids and have a lower melting point than triacylglycerols from the inner layer (15). The ram fats in this study showed similar differences, although of greater magnitude for 18:0 and 18:1, to those of the pig. However, the ram fats contained appreciable amounts of branched-chain acids that were not present in the pig fats, possibly reflecting the different digestive systems of these two animals. These acids arise from the biosynthetic incorporation of methylmalonate (derived from propionate), rather than malonate (derived from acetate), into the growing fatty acid chains (5).

In sheep (23) and in pigs (24), both the liver and adipose tissue are considered as important sites for the synthesis of fatty acids from acetate. The formation of the soft outer subcutaneous fat layer in pigs has been suggested to involve desaturation of saturated components in this layer, rather than transport of unsaturated components from some other site (18). Similarly, an enhanced synthesis of unsaturated and branched-chain acids may take place in the soft outer region of the subcutaneous fat of rams.

REFERENCES

1. L'Estrange, J.L., and Mulvihill, T.A. (1975) *J. Agric. Sci., Camb. 84,* 281–290.
2. Duncan, W.R.H., Orskov, E.R., and Garton, G.A. (1972) *Proc. Nutr. Soc. 31,* 19A–20A.
3. Miller, G.J., Varnell, T.R., and Rice, R.W. (1967) *J. Anim. Sci. 26,* 41–45.
4. Miller, G.J., and Rice, R.W. (1967) *J. Anim. Sci. 26,* 1153–1159.
5. Garton, G.A., Hovell, F.D. DeB., and Duncan, W.R.H. (1972) *Br. J. Nutr. 28,* 409–416.
6. Purchas, R.W., O'Brien, L.E., and Pendleton, C.M. (1979) *N.Z. J. Agric. Res. 22,* 375–383.
7. Miller, G.J., Kunsman, J.E., and Field, R.A. (1980) *J. Food Sci. 45,* 279–282.
8. Busboom, J.R., Miller, G.J., Field, R.A., Crouse, J.D., Riley, M.L., Nelms, G.E., and Ferrell, C.L. (1981) *J. Anim. Sci. 52,* 83–92.
9. Christie, W.W. (1982) *Lipid Analysis,* 2nd edition, Pergamon Press, Oxford, England.
10. Official and Tentative Methods of the American Oil Chemists' Society, 3rd edn., revised to 1984, AOCS, Champaign, IL.
11. van Wijngaarden, D. (1967) *Anal. Chem. 39,* 848–849.
12. Johnson, C.B., Wong, E., Birch, E.J., and Purchas, R.W. (1977) *Lipids 12,* 340–347.
13. Shorland, F.B., Czochanska, Z., Barton, R.A., and Rae, A.L. (1967) *J. Agric. Sci., Camb. 68,* 221–225.
14. Duncan, W.R.H., Lough, A.K., Garton, G.A., and Brooks, P. (1974) *Lipids 9,* 669–673.
15. Christie, W.W., Jenkinson, D. McE., and Moore, J.H. (1972) *J. Sci. Food Agric. 23,* 1125–1129.
16. Drury, R.A.B., and Wallington, E.A. (1967) *Carleton's Histological Technique,* 4th edn., Oxford University Press, New York, NY.
17. Hilditch, T.P., and Williams, P.N. (1964) *The Chemical Constitution of Natural Fats,* Chapman and Hall, London, England.
18. Hansen, R.P., and Czochanska, Z. (1976) *N.Z. J. Sci. 19,* 413–419.
19. Leat, W.M.F. (1975) *J. Agric. Sci., Camb. 85,* 551–558.
20. Kuksis, A. (1978) in *Handbook of Lipid Research, Fatty Acids and Glycerides* (Kuksis, A., ed.) Vol. 1, pp. 381–442, Plenum Press, New York, NY.
21. Markley, K.S. (1960) in *Fatty Acids. Their Chemistry, Properties, and Uses* (Markley, K.S., ed.) Part 1, pp. 23–247, Interscience Publishers, New York, NY.
22. Weast, R.C., ed. (1976) *Handbook of Chemistry and Physics,* 57th edn., CRC Press Inc., Boca Raton, FL.
23. Hanson, R.W., and Ballard, F.J. (1967) *Biochem. J. 105,* 529–536.
24. O'Hea, E.K., and Leveille, G.A. (1969) *J. Nutr. 99,* 338–344.

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Effect of Various Steroids on the Biosynthesis of Arachidonic Acid in Isolated Hepatocytes and HTC Cells

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The effect of various steroids on the incorporation and desaturation of eicosa-8,11,14-trienoic acid in normal hepatocytes and HTC cells was investigated. After 3 hr incubation with 11-deoxycorticosterone, both kinds of cells showed an increase in the incorporation of eicosatrienoic acid. In contrast, progesterone, cortexolone, 17-β-estradiol, testosterone, estriol, aldosterone, corticosterone, dexamethasone, dehydroepiandrosterone, 11-β-hydroxyandrosterone, 11-ketoaetiocholanolone, epiaetiocholanolone and 5-β-pregnane-3α,20α-diol, provoked no significant changes in the uptake of the exogenous acid. Of all the steroids tested, only 11-dexycorticosterone, dexamethasone and 17-β-estradiol evoked a significant inhibition on the arachidonate biosynthesis in both kinds of cells. Testosterone, estriol, aldosterone and corticosterone provoked a significant inhibition of Δ5-desaturase in HTC cells. In dexamethasone, this effect was dose-dependent (0 to 10^{-4} M). Simultaneous incubation with 17-β-estradiol or 11-deoxycorticosterone with dexamethasone led to an extent of inhibition on arachidonate biosynthesis that did not surpass the effect of each drug. Pretreatment of isolated hepatocytes with the antiglucocorticoid, cortexolone, prevented the dexamethasone-induced inhibition of arachidonate biosynthesis. Normal rat liver microsomes preincubated in vitro with dexamethasone, 11-deoxycorticosterone, 17-β-estradiol, corticosterone or estriol (10^{-6} or 10^{-4} M concentration), showed no significant changes in the Δ5-desaturase activity. The results obtained suggest that the effect of the steroids on arachidonic acid biosynthesis in normal hepatocytes and HTC cells requires receptor occupancy and probably is mediated through a common biochemical mechanism.
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Previous works have demonstrated that oral administration or intraperitoneal injection of glucocorticoid hormones in rats produced a decrease in the activities of Δ6- and Δ5-liver-microsomal desaturases (1,2). In addition, recent studies performed in our laboratory have revealed that the incubation of isolated hepatocytes or HTC cells (the permanent cell-culture line, hepatoma tissue culture 7288c) with dexamethasone also produced a decrease in the conversion of eicosa-8,11,14-trienoic acid to arachidonic acid (3). The mechanism by which dexamethasone exerts this biological effect is still unknown. However, as previously reported (3), the modulatory action of this class of hormone on desaturase activity would be expected to be produced through a mechanism similar to that mediating the other known metabolic effect of glucocorticoids (4–9), namely through induction of the biosynthesis of a regulatory protein. Classically, steroid hormones trigger their effects after combining with a high-affinity cytosolic receptor (6). A property common to all steroid receptors is their ability to recognize subtle differences in steroidal molecular structure. Within this context, the aim of the present work was to test the effect of different steroid hormones on the incorporation and conversion of eicosatrienoic acid in isolated cells. In this system, we have studied four different classes of steroid hormones: glucocorticoids, mineralocorticoids, estrogens and androgens. Also tested was cortexolone that competitively inhibits the action of glucocorticoids, and it is known as an antiinducer (10–13).

MATERIAL AND METHODS

Chemicals. [1-^{14}C]Eicosa-8,11,14-trienoic acid (54.9 mCi/mmol, 98% radiochemically pure) was provided by New England Nuclear Corp. (Boston, MA). Unlabeled eicosa-8,11,14-trienoic acid was supplied by Nu-Chek Prep (Elysian, MN). Bovine serum albumin, essentially fatty acid-free bovine albumin, N-2-hydroxy-ethyl-piperazine-N-2-ethanol sulphonic acid (HEPES), collagenase type IV and trypsin soybean inhibitor type I were provided by Sigma Chemical Company (St. Louis, MO). Dexamethasone was obtained from Merck, Sharp & Dohme Laboratories (Buenos Aires, Argentina). Dehydroepiandrosterone was purchased from Merck Laboratories (Darmstadt, Germany). 17-β-Estradiol was provided by Gador Laboratories (Buenos Aires, Argentina) and 11-deoxycorticosterone was supplied by Schering Laboratories (Buenos Aires, Argentina). All other steroids were purchased from Sigma Chemical Company (St. Louis, MO).

Animals. Adult female Wistar rats weighing 160–180 g fed on a standard pelleted diet (Cargill, Rosario, Argentina) were used. In calories, this standard diet consisted of 56.7% carbohydrates, 10.4% lipids and 32.9% protein, vitamins and minerals. Water was given ad libitum. In order to avoid individual differences among the animals, in all the experiments the rats were fasted for 24 hr, refed for 2 hr and then killed 12 hr after the end of the feeding period.

Isolation of microsomes. Livers were rapidly excised and immediately placed in ice-cold homogenizing medium (14). They were homogenized in 3 ml of homogenizing solution for each gram of liver tissue and then centrifuged for 30 min at 10,000 × *g*. The pellet was discarded and the supernatant fraction was centrifuged again for 60 min

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Abbreviations: Aldosterone, 4-pregnen-18-al-11β,21-diol-3,20-dione; cortexolone, 17-α,21-dihydroxy-4-pregnene-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; dehydroepiandrosterone, 5-androsten-3β-ol-17-one; 17-β-estradiol, 1,3,5[10]-estratriene-3,17-β-diol; 11-deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; dexamethasone, 1,4-pregnadien-9-α-fluoro-16α-methyl-11β, 17-α,21-trihydroxy-3,20-dione; epiaetiocholanolone, 5β-androstan-3β-ol-17-one; estriol,1,3,5-[10]estratriene-3,16α,17β-triol; HEPES, N-2-hydroxy-ethyl-piperazine-N-2-ethanol sulphonic acid; 11-β-hydroxyandrosterone, 5-α-androstan-3α-11β-diol-17-one; 11-ketoaetiocholanolone, etiocholan-3α-ol-11,17-dione; 5β-pregnane-3α,20α-diol,3α,20α-dihydroxy-5β-pregnane; progesterone, 4-pregnene-3,20-dione; testosterone, 4-androsten-17β-ol-3-one; HTC, hepatoma tissue culture.

at 110,000 × *g* in a Spinco L-2 ultracentrifuge. The pellets were resuspended in cold homogenizing solution up to a final protein concentration of 50 mg per ml and were used immediately for fatty acid desaturase activity assay.

Incubation of rat liver microsomes with different steroid hormones. A possible direct effect of different steroidal compounds on the microsomal Δ5-desaturation system was examined. The microsomal suspension was preincubated for 15 min at 37°C with dexamethasone, 11-deoxycorticosterone, 17-β-estradiol, corticosterone or estriol at 10^{-6} and 10^{-4} M concentrations. Control tubes were preincubated with the vehicle in which steroid hormones were dissolved (ethanol 20 mM). At the end of the preincubation period, Δ5-desaturase activity was measured by estimation of the percentage conversion of [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid. Five nmol of the labeled acid and 95 nmol of unlabeled acid were incubated with 5 mg of microsomal protein in a shaker at 37°C for 10 min. The composition of the incubation medium, the procedure to obtain the fatty acid methyl esters and the quantitative analysis of radioactive fatty acid methyl esters, have been described elsewhere (15).

Isolated cells. Liver parenchymal cell isolation from Wistar rats was carried out according to Seglen (16), except that the operational temperature was maintained at 37°C. To minimize glycogenolysis, 0.2% glucose was added to all media used during perfusion and isolation (17). The liver cells were suspended in oxygenated Hank's buffer (18) containing 1.0 g % of bovine albumin. After 30 min, the cells were centrifuged and resuspended in IMEM-Zo medium (19), minus linoleic acid and containing HEPES (5 mM). HTC cells were established in culture from an ascites tumor originally derived from a solid hepatoma (7288 c) (20). The cells were grown in surface culture at 37°C, under sterile conditions, in Swim's 77 medium (S-77) supplemented with 10% (v/v) calf serum (21) and were harvested at confluence. The suspensions of HTC cells were obtained from surface cultures as described elsewhere (22). Cell viability (90%) was assessed by the criterion of trypan blue dye exclusion (23).

Experimental procedures with isolated cells. HTC cells and isolated liver cells were counted in a hemocytometer. Aliquots (3.0×10^6 cells) were incubated with 5 ml of modified IMEM-Zo medium in 25-ml siliconized Erlenmeyer flasks under an atmosphere of 95% oxygen and 5% carbon dioxide in a metabolic shaker (70 rpm) at 37°C. To measure the incorporation and conversion of exogenous eicosa-8,11,14-trienoic acid in both kinds of cells after treatment with different steroid hormones, we performed four types of experiments.

In the first series, HTC cells or normal hepatocytes were incubated for 3 hr with different steroid hormones at a concentration of 0.1, 1.0 or 10 mM. Twenty min before the end of the incubation period, all media were supplemented with a mixture of labeled eicosa-8,11,14-trienoic acid at a final concentration of 80 μM (0.5 μCi per flask). The acid was added as its sodium salt bound to albumin delipidated according to Spector et al. (24).

In the second experiment, incubation of the cells was carried out in an identical manner, but with dexamethasone (0.1 mM) as the active glucocorticoid either alone or in combination with 11-deoxycorticosterone (0.1 mM) or 17-β-estradiol (10 mM).

In the third set of experiments, isolated normal hepatocytes were incubated with dexamethasone (10^{-6} or 10^{-4} M) either alone or in combination with cortexolone (10^{-6} or 10^{-4} M). Here, after 1 hr incubation with cortexolone, dexamethasone was added to some flasks and the cells were incubated with the two hormones for another 3 hr. Then, eicosa-8,11,14-trienoic acid was added to all the flasks, as in the first experiment.

To determine the dose-response curve for inhibition of arachidonate biosynthesis in HTC cells, another type of experiment was carried out. HTC cells were incubated for 3 hr with dexamethasone phosphate at different concentrations (ranging from 0 to 10^{-4} M). Then, eicosatrienoic acid was added to all the flasks, as in the first experiment.

At the end of the incubation periods, cell suspensions were transferred to ice-cold tubes and centrifuged. Each pellet was washed three times with 0.85 g % NaCl (3 ml each time) and resuspended (5 ml) in the same solution. An aliquot of the suspension was used to determine cell viability (23) and the amount of cellular protein (25), and the rest was centrifuged at 2000 × *g* for 5 min. The saline solution was decanted and the cell pellets were saponified for 45 min at 85°C with 2.0 ml of 10% (w/v) KOH in ethanol plus 500 μl of methanol to facilitate lipid extraction. The free fatty acids were extracted from the acidified solution with light petroleum (bp 30–40°C) and esterified with 3 N HCl in methanol for 3 hr at 65°C.

Chromatographic measurements. The radioactivity of the recovered methyl esters (usually 96–98%) was measured in a Beckman liquid-scintillation counter (model LS-3133 P) with 96% efficiency for ^{14}C. The distribution of radioactivity among the fatty acid methyl esters was determined by gas-liquid radiochromatography using an Acromat CG-100 equipped with a Packard proportional counter. Percentage conversion of eicosa-8,11,14-trienoic acid to arachidonic acid was calculated from the distribution of radioactivity between the substrate and product measured directly on the radiochromatogram (26). The column was packed with 10% SP-2330 coated on 100–200 chromosorb WAW (Supelco, Bellefonte, PA). Identification of methyl esters was made by comparison with known standards. Results were calculated as mean ± 1 SEM; statistical analyses were made using the Student-t-test.

RESULTS

We first examined the effect of different steroid hormones on the incorporation and desaturation of eicosa-8,11,14-trienoic acid in isolated rat liver cells (Fig. 1). After 3 hr incubation in the presence of 11-deoxycorticosterone (0.1 mM), a significant increase in the incorporation of the acid was observed (ca. 40% over control values). The uptake of the exogenous eicosatrienoic acid was not modified by incubating the cells in the presence of the other steroid hormones (dexamethasone, progesterone, cortexolone or 17-β-estradiol).

Of all the hormones tested and indicated in Figure 1, only dexamethasone, 11-deoxycorticosterone and 17-β-estradiol had a significant effect on the conversion of eicosatrienoic acid to arachidonic acid in isolated hepatocytes. The inhibition of arachidonic acid biosynthesis produced by dexamethasone was less potent (48% at 0.1 mM) than those produced by 11-deoxycorticosterone (61% at

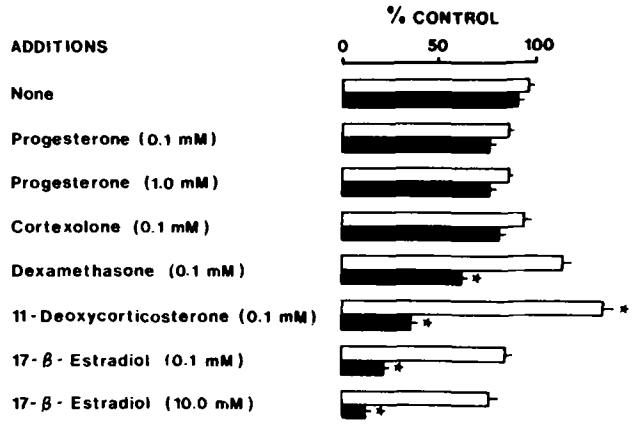

FIG. 1. Effect of different steroid hormones on the incorporation (□) and desaturation (■) of [1-^{14}C]eicosatrienoic acid in isolated rat hepatocytes. Cell suspensions were incubated for 3 hr with or without each of the steroid hormones at the concentrations listed in the figure. The results were expressed as percentage change from control flasks incubated for 20 min with labeled acid 80 μM concentration (42.5 ± 1.3 nmol of eicosatrienoic acid incorporated and 3.0 ± 0.2 nmol of arachidonic acid formed, per mg of cellular protein). Conditions of incubation are described in experimental procedures. Values are the mean of 3 incubation flasks. Vertical lines represent 1 SEM. Results significantly different, $P < 0.01$, are indicated with a star.

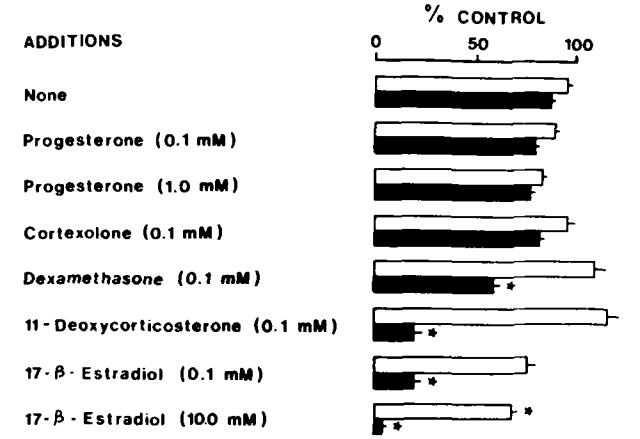

FIG. 2. Effect of different steroid hormones on the incorporation (□) and desaturation (■) of [1-^{14}C]eicosatrienoic acid in HTC cells. Cell suspensions were incubated for 3 hr with or without each of the steroid hormones at the concentrations listed in the figure. The results were expressed as percentage change from control flasks incubated for 20 min with labeled acid 80 μM concentration (25.2 ± 0.8 nmol of eicosatrienoic acid incorporated and 4.3 ± 0.1 nmol of arachidonic acid formed, per mg of cellular protein). Conditions of incubation are described in experimental procedures. Values are the mean of 3 incubation flasks. Vertical lines reprsent 1 SEM. Results significantly different, $P < 0.01$, are indicated with a star.

0.1 mM) and 17-β-estradiol (75% and 88% at 0.1 to 10.0 mM, respectively.

Similar results were obtained by incubating HTC cells in the presence of the same steroid hormones (Fig. 2). In this experiment, however, 11-deoxycorticosterone had no significant effect on the incorporation of eicosatrienoic acid, but 10.0 mM 17-β-estradiol caused a marked decrease in both the incorporation of the acid (30%) and its conversion to arachidonic acid (94%).

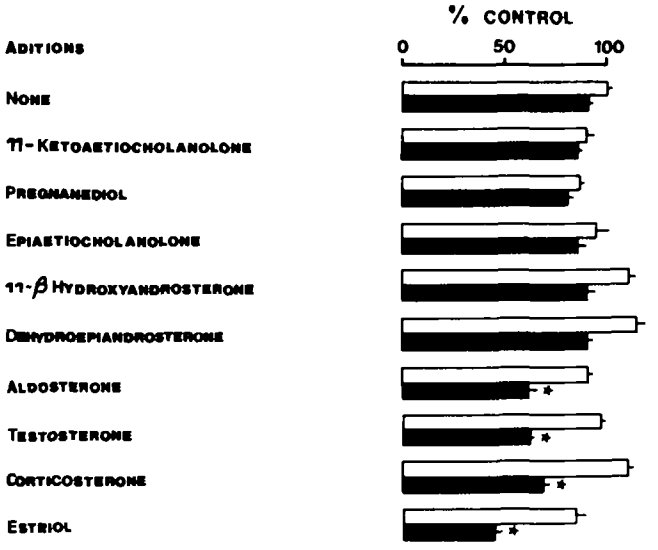

FIG. 3. Effect of different steroid compounds on the incorporation (□) and desaturation (■) of [1-^{14}C]eicosatrienoic acid in hepatoma cells. HTC cell suspensions were incubated for 3 hr with or without each of the steroid hormones at 0.1 mM concentration. The results were expressed as percentage change from control flasks incubated for 20 min with labeled acid 80 μM concentration (24.7 ± 0.9 nmol of eicosatrienoic acid incorporated and 4.8 ± 0.3 nmol of arachidonic acid formed, per mg of cellular protein). Conditions of incubation are described in experimental procedures. Values are the mean of 3 incubation flasks. Vertical lines represent 1 SEM. Results significantly different, $P < 0.01$, are indicated with a star.

We also examined the effect of other steroids on the uptake and desaturation of eicosatrienoic acid in the hepatoma cells (Fig. 3). None of the hormones studied (11-ketoaetiocholanolone, 5-β-pregnane-3α,20α-diol, epiaetiocholanolone, 11-β-hydroxyandrosterone, dehydroepiandrosterone, aldosterone, testosterone, corticosterone and estriol) affected the incorporation of the acid, although the biosynthesis of arachidonate was significantly depressed by aldosterone, testosterone, corticosterone and estriol at a concentration of 0.1 mM.

In order to reveal a possible competitive effect between different steroid hormones, 11-deoxycorticosterone or 17-β-estradiol was added to the incubation medium at the same time as dexamethasone. The results obtained with dexamethasone in combination with either of the other two steroids in isolated hepatocytes (Fig. 4) or HTC cells (Fig. 5) were similar to earlier observations made with those hormones alone in the corresponding cell types (Figs. 1 and 2, respectively).

Short-term treatment of isolated hepatocytes with either progesterone or cortexolone failed to modify either of the parameters studied (Fig. 6). Moreover, pretreatment of the cells for 1 hr with antiglucocorticoid (10^{-4} M) prevented the subsequent inhibition by dexamethasone (10^{-4} or 10^{-6} M), otherwise seen in this system.

A final experiment with HTC cells (Fig. 7) demonstrated that the dexamethasone effect on arachidonate production was dose-dependent.

When rat liver microsomal fractions were preincubated at 37°C for 15 min with dexamethasone, 11-deoxycorticosterone, 17-β-estriol, aldosterone or estriol (10^{-6} or 10^{-4} M concentration) and compared to microsomes

preincubated in hormone absence, no significant changes in the Δ5-desaturation activity were observed (Table 1)

DISCUSSION

At the time of these studies, little was known about the role of different steroid hormones in the regulation of polyunsaturated fatty acid metabolism. Previous studies

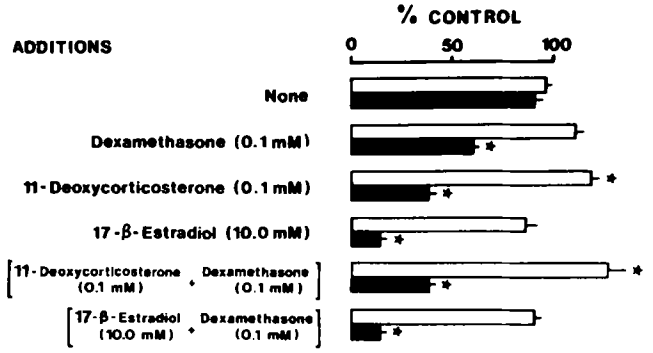

FIG. 4. Competitive effect of steroid hormones on the incorporation of [1-^{14}C]eicosatrienoic acid (□) and its conversion to arachidonate (■) in isolated hepatocytes of rats. Normal liver cells were incubated for 3 hr with dexamethasone (0.1 mM) either alone or in combination with 11-deoxycorticosterone (0.1 mM) or 17-β-estradiol (10.0 mM). Some flasks were also supplemented with the latter two steroid hormones at the concentrations indicated in the figure. Twenty min before the end of the incubation period, all media were supplemented with labeled eicosatrienoic acid (80 μM) as it was described in experimental procedures. The results were expressed as percentage change from control flasks incubated for 20 min with the acid (39.4 ± 1.1 nmol of eicosatrienoic acid incorporated and 2.8 ± 0.1 nmol of arachidonic acid formed, per mg cellular protein). Bars and vertical lines represent means and SEM of 3 experiments. Results significantly different, $P < 0.01$, are indicated with a star.

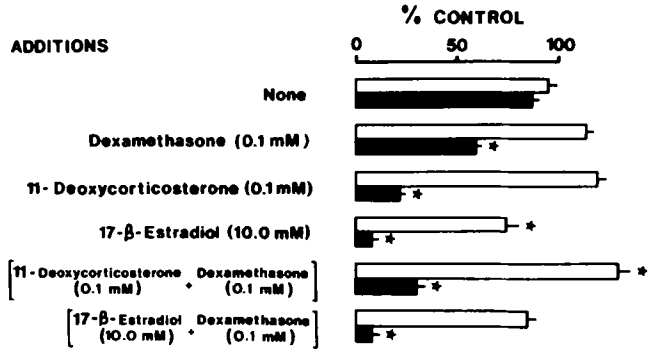

FIG. 5. Competitive effect of steroid hormones on the incorporation of [1-^{14}C]eicosatrienoic acid (□) and its conversion to arachidonate (■) in HTC 7288c cells. The cells were incubated for 3 hr with dexamethasone (0.1 mM) either alone or in combination with 11-deoxycorticosterone (0.1 mM) or 17-β-estradiol (10.0 mM). Some flasks were also supplemented with the latter two steroid hormones at the concentrations indicated in the figure. Twenty min before the end of the incubation period, all media were supplemented with labeled eicosatrienoic acid (80 μM) as it was described in experimental procedure. The results were expressed as percentage change from control flasks incubated for 20 min wth the acid (25.1 ± 0.6 nmol of eicosatrienoic acid incorporated and 3.9 ± 0.1 nmol of arachidonic acid formed, per mg of cellular protein). Bars and vertical lines represent means and SEM of 3 experiments. Results significantly different, $P < 0.01$, are indicated with a star.

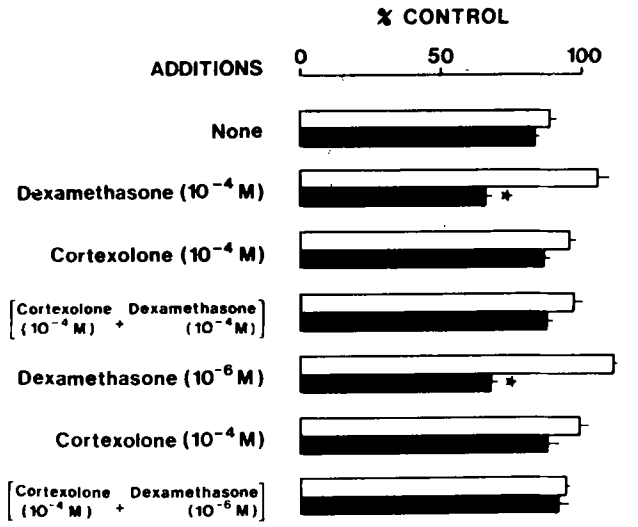

FIG. 6. Effect of pretreatment of isolated liver cells with antiglucocorticoids on the action of dexamethasone on eicosatrienoic acid incorporation (□) and desaturation (■). Normal isolated hepatocytes were incubated for 4 hr with dexamethasone (10^{-6} or 10^{-4} M) either alone or in combination with cortexolone (10^{-6} or 10^{-4} M). In these experiments, after 1 hr of incubation with the antiglucocorticoid cortexolone, dexamethasone was added to some flasks and the cells were incubated with the two hormones for another 3 hr. Labeled eicosatrienoic acid (80 μM final concentration) was added to all the flasks 20 min before the end of the incubation period. The results were expressed as percentage change from control cells incubated for 20 min with the acid (37.5 ± 0.9 nmol of eicosatrienoic acid incorporated and 3.1 ± 0.2 nmol of arachidonic acid formed, per mg of cellular protein). Bars and vertical lines represent means and SEM of 3 experiments. Results significantly different, $P < 0.01$, are indicated with a star.

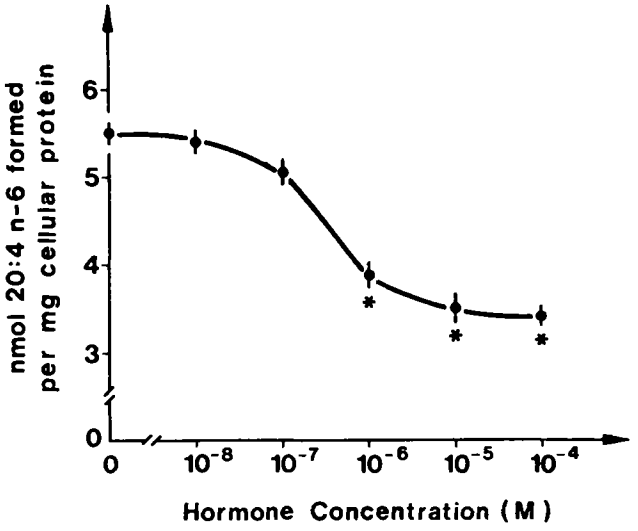

FIG. 7. Dose-response curve for inhibition of arachidonate biosynthesis in HTC cells by dexamethasone phosphate (0 to 10^{-4} M concentration). The cells were incubated at 37°C for 3 hr with the steroid hormone. Twenty min before the end of incubation period, the media were supplemented with labeled eicosatrienoic acid (80 μM concentration) as it was described in experimental procedure. The results were expressed as nmol of arachidonic acid formed per mg of cellular protein. Vertical lines represent SEM of 3 experiments. Results significantly different, $P < 0.01$, are indicated with a star.

TABLE 1

Direct Effect of Steroids on Microsomal Rat Liver Δ5-Desaturase Activity

Treatment	% Conversion 20:3 n-6 to 20:4 n-6
None	40.9 ± 1.3
Dexamethasone (10^{-6} M)	38.2 ± 0.4
Dexamethasone (10^{-4} M)	37.7 ± 0.2
11-Deoxycorticosterone (10^{-6} M)	39.1 ± 1.2
11-Deoxycorticosterone (10^{-4} M)	38.4 ± 0.5
17-β-Estradiol (10^{-6} M)	42.4 ± 2.3
17-β-Estradiol (10^{-4} M)	37.8 ± 0.8
Aldosterone (10^{-6} M)	39.7 ± 1.8
Aldosterone (10^{-4} M)	38.1 ± 1.7
Estriol (10^{-6} M)	41.4 ± 0.5
Estriol (10^{-4} M)	39.5 ± 1.5

Five mg of microsomal protein were preincubated at 37°C for 15 min with the steroids indicated. Control tubes were preincubated with ethanol 20 mM. At the end of the preincubation period, Δ5-desaturase activity was determined as it was stated in Materials and Methods. The results correspond to the percentage conversion of eicosa-8,11,14-trienoic acid to arachidonic acid and they are the mean ±1 SEM of 3 experiments.

from this laboratory had shown that the administration of natural or synthetic glucocorticoids to rats (hydrocortisone, dexamethasone or triamcinolone) produced a decrease in liver microsomal Δ6- and Δ5-desaturase activities (1). In reports by others, the injection of 17-β-estradiol to ovariectomized rats had depressed Δ6-desaturase (27), but the treatment of roosters with the same hormone had stimulated Δ9-desaturase (28).

In this paper, we have demonstrated that, among a variety of steroid hormones of widely different structures (glucocorticoids, mineralocorticoids, androgens and estrogens), only 11-deoxycorticosterone was able to increase significantly the incorporation of eicosatrienoic acid to cells, and 17-β-estradiol evoked a depressive effect. The effect of 11-deoxycorticosterone on the incorporation of eicosatrienoic acid and its conversion to arachidonic acid were opposite, as a significant increase in the incorporation of the acid was seen with a simultaneous decrease in the biosynthesis of arachidonic acid (Fig. 1).

The inhibition of the Δ5-desaturase was more pronounced with 11-deoxycorticosterone and 17-β-estradiol than with dexamethasone, aldosterone, testosterone, corticosterone and estriol. We also found no additive effect between dexamethasone and either 11-deoxycorticosterone or 17-β-estradiol with respect to the inhibition of eicosatrienoic acid desaturation in either isolated hepatocytes (Fig. 4) or HTC cells (Fig. 5). The mechanism through which glucocorticoids modify the desaturation of fatty acid can be explained in different ways. It could be genomic or nongenomic. A possible nongenomic mechanism of dexamethasone action could be evoked by direct binding of the hormone to the microsomes, as already reported for rat liver (29). This effect of the hormone could be produced directly on the Δ5-desaturation system or mediated through changes of the physicochemical properties of the biomembrane that, as we know, may modulate desaturase activity (30,31). However, the preincubation of rat liver microsomes with dexamethasone, 11-deoxycorticosterone, 17-β-estradiol, corticosterone or estriol (10^{-6} or 10^{-4} M concentration) did not produce any significant and specific effect on the Δ5-desaturase activity (Table 1). Therefore, we may discard rather confidently a direct effect of steroids on Δ5-desaturation system.

Regarding the classical mechanism of action generally accepted, steroids enter the cells by simple diffusion and combine with a high affinity receptor (32,33). That the dexamethasone inhibition of eicosatrienoic acid desaturation was blocked by pretreatment of the isolated hepatocytes with antiglucocorticoid (Fig. 6) is consistent with the hypothesis that the effects of the steroid in this biological and biochemical system are mediated through receptor occupancy. The fact that dexamethasone phosphate produced an inhibition of arachidonic acid biosynthesis in HTC cells in a dose-dependent fashion (Fig. 7) also suggests that glucocorticoid receptor structures would be involved.

Based on the above observations together with other evidence obtained in our laboratory (3,34), we can now conclude that the inhibition by steroid hormones of arachidonate biosynthesis does not result from a direct interaction with the Δ5-desaturase enzymatic machinery, but rather requires initial cytoplasmic-receptor occupancy, as well as likely subsequent changes in gene transcription and/or translation. Moreover, these data would also suggest that, despite their differing molecular structures, several steroid hormones inhibit eicosatrienoic acid desaturation probably through a common biochemical mechanism. Further research, however, is needed to evaluate the possible role of these hormones in controlling the activity of desaturases other than the Δ5.

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REFERENCES

1. de Gómez Dumm, I.N.T., de Alaniz, M.J.T., and Brenner, R.R. (1979) *J. Lipid Res. 20,* 834-839.
2. Mandon, E.C., de Gómez Dumm, I.N.T., de Alaniz, M.J.T., Marra, C.A., and Brenner, R.R. (1987) *J. Lipid Res. 28,*1377-1383.
3. Marra, C.A., de Alaniz, M.J.T., and Brenner, R.R. (1986) *Lipids 21,* 212-219.
4. Flower, R.J., and Blackwell, G.J. (1979) *Nature 278,* 456-459.
5. Hirata, F., Schiffmann, E., Venkatasubramanian, K., Salomon, D., and Axelrod, J. (1980) *Proc. Natl. Acad. Sci. USA 77,* 2533-2536.
6. Duval, D., Durant, S., and Homo-Delarche, F. (1983) *Biochim. Biophys. Acta 737,* 409-442.
7. Cwikel, B.J., Barouski-Miller, P.A., Coleman, P.L., and Gelehrter, T.D. (1984) *J. Biol. Chem. 259,* 6847-6851.
8. Di Rose, M., Flower, R.J., Hirata, F., Parente, L., and Russo-Marie, F. (1984) *Prostaglandins 28,* 441-442.
9. Errasfa, M., Rothut, B., and Fradin, A.F. (1985) *Biochim. Biophys. Acta 847,* 247-254.
10. Makman, M.H., Nakagawa, S., and White, A. (1967) *Recent Prog. Hormone Res. 23,* 195-218.
11. Simonsson, B. (1972) *Acta Physiol. Scand. 86,* 398-409.
12. Kaiser, N., Milholland, R.J., Turnell, R.W., and Rosen, F. (1972) *Biochem. Biophys. Res. Commun. 49,* 516-521.

13. Kaiser, N., Solo, A.J., Milholland, R.J., and Rosen, F. (1974) *J. Biol. Chem. 249,* 1133-1138.
14. Castuma, J.C., Catalá, A., and Brenner, R.R. (1972) *J. Lipid Res. 21,* 212-219.
15. de Gómez Dumm, I.N.T., de Alaniz, M.J.T., and Brenner, R.R. (1978) *Lipids 13,* 649-652.
16. Seglen, R.O. (1973) *Exp. Cell Res. 84,* 391-398.
17. Harris, R.A. (1975) *Arch. Biochem. Biophys. 169,* 168-180.
18. Hanks, J.H., and Wallace, R.E. (1949) *Proc. Soc. Exp. Biol. Med. 71,* 196-200.
19. Richter, H., Sanford, K.K., and Evans, V.J. (1972) *J. Natl. Cancer Inst. 49,* 1705-1712.
20. Thompson, E.B., Tomkins, G.N., and Curran, J.F. (1966) *Proc. Natl. Acad. Sci. USA 56,* 296-303.
21. de Alaniz, M.J.T., Ponz, G., and Brenner, R.R. (1975) *Acta Physiol. Latinoam. 25,* 1-11.
22. Marra, C.A., de Alaniz, M.J.T., and Brenner, R.R. (1985) *Mol. Cell. Biochem. 65,* 143-152.
23. Jauregui, H.O., Ayner, N.T., Driscoll, J.L., Williams-Hollans, H., Lipsky, N.H., and Galleti, B.M. (1981) *In Vitro 17,* 1100-1110.
24. Spector, A.A., Steinberg, D.S., and Tanaka, A. (1965) *J. Biol. Chem. 240,* 1032-1041.
25. Lowry, O.H., Rosebrough, M.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 275-295.
26. Brenner, R.R., and Peluffo, R.O. (1966) *J. Biol. Chem. 241,* 5213-5219.
27. González, S., Nervi, A.M., and Peluffo, R.O. (1986) *Lipids 21,* 440-443.
28. Lippiello, P.M., Holloway, C.T., Gardfield, S.A., and Holloway, P.W. (1979) *J. Biol. Chem. 254,* 2004-2009.
29. Onrani, G.R., Furukawa, H., Sherwood, J.A., and Loeb, J.N. (1983) *Endocrinology 112,* 178-186.
30. Garda, H.A., and Brenner, R.R. (1984) *Biochim. Biophys. Acta 768,* 160-170.
31. Garda, H.A., and Brenner, R.R. (1986) *Biochim. Biophys. Acta 819,* 45-54.
32. Russo-Marie, F., and Duval, D. (1982) *Biochim. Biophys. Acta 712,* 177-185.
33. Duval, D., Durant, S., and Homo-Delarche, F. (1983) *Biochim. Biophys. Acta 737,* 409-442.
34. Marra, C.A., de Alaniz, M.J.T., and Brenner, R.R. (1986) *Biochim. Biophys. Acta 879,* 388-393.

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A Diet Containing n-3 and n-6 Fatty Acids Favorably Alters the Renal Phospholipids, Eicosanoid Synthesis and Plasma Lipids in Nephrotic Rats

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The nephrotic syndrome was induced in rats by intravenous adriamycin (3 mg/kg). The rats were then divided into four groups which, for six weeks, were pair-fed diets containing beef tallow (BT), fish oil (FO), a source of n-3 fatty acids, evening primrose oil (EPO), a source of n-6 fatty acids, or a combination of evening primrose oil and fish oil, 75:25 (EPO:FO). The fat content of the diets was 15%. Significant incorporation of the fatty acids into kidney phospholipids was demonstrated. Diets containing FO, EPO and EPO:FO lowered plasma triglycerides and total cholesterol levels as compared with diets containing BT. Only EPO:FO raised high density lipoprotein (HDL) cholesterol levels, as compared with BT. The combination EPO:FO prevented the tenfold suppression of aortic 6-keto-$PGF_{1\alpha}$ caused by FO. These changes in plasma lipids and eicosanoid production are potentially antiatherogenic and may prevent glomerular sclerosis. The combination of EPO and FO, containing n-6 and n-3 fatty acids may offer advantages over either family of fatty acids in this model of nephrotic syndrome.
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Diets containing fish oil (FO) and rich in n-3 polyunsaturated fatty acids (PUFA), lower plasma lipids and preserve kidney function and histology in adriamycin-induced nephrotic rats (1,2). Fish oil suppresses platelet and glomerular thromboxane A_2 (TXA_2), a beneficial effect, but also suppresses aortic production of the vasodilatory prostaglandin I_2 (PGI_2) and glomerular production of PGE and PGI_2. This is an undesirable action. Evening primrose oil (EPO) contains the n-6 PUFA gammalinolenic and linoleic acids; both are precursors of monoenoic and dienoic eicosanoids through the cyclooxygenase pathway. We tested the hypothesis that the addition of n-6 PUFA to a fish-oil diet may prevent suppression of beneficial PG by increasing their precursors in the cell phospholipids. The effect of a mixture of EPO and FO (75:25) was studied. The results were compared with those in animals fed beef tallow (BT), EPO or FO alone as dietary fats. In contrast with previous studies, we used a dose of adriamycin of 3 mg/kg instead of 7.5 mg/kg, to prevent development of advanced renal disease and renal failure, which could interfere with the variables measured.

MATERIAL AND METHODS

Animals and diets. Forty-one Sprague-Dawley female rats (Harlan, Indianapolis, IN) weighing 220–270 g received a single dose of adriamycin (3 mg/kg) (Adria Laboratories, Columbus, OH) into the tail vein, under ether anesthesia. Seven days later (week 0), matched by weight and urinary protein excretion, groups of rats were allocated to four isocaloric diets, containing 14% BT, 14% FO, 14% EPO and 14% of a mixture of EPO and FO, 75:25 (EPO:FO). Soybean oil (SBO) 1% was added to all diets to supplement essential fatty acids. EPO, FO and EPO:FO were supplied by Efamol, Inc. (Kentville, Nova Scotia, Canada). The diets were prepared by Teklad (Madison, WI), who also provided BT and SBO fats. All experimental diets contained 200 g/kg protein as vitamin-free casein, 3 g/kg DL methionine, 382 g/kg sucrose, 150 g/kg maltodextrin, 150 g/kg fat, 70 g/kg cellulose, 35 mg/kg mineral mix (Teklad AIN-76), 10 g/kg vitamin mix (Teklad #40060) that provides 0.24 g/kg diet of dry vitamin-E acetate, and 26 mg/kg of BHA (an antioxidant). The fatty acid composition of the dietary lipids, analyzed by gas chromatography (GC) is shown in Table 1. Pair-feeding was used throughout, with the rats in three groups being pair-fed against the rats in the group with the lowest food intake.

Rats were weighed periodically and kept without food but with free access to water. Urine was collected for 23 hr, at weeks 0 and 4. Systolic blood pressure was measured by the tail cuff method at week 5. At week 6, after a 15 hr fast, rats were killed under ketamine-xylazine anesthesia by exsanguination via cardiac puncture. Plasma creatinine, lipids and platelet TXA_2 production were measured. Kidney tissue was obtained for fatty acid composition, light microscopy and eicosanoid production.

Glomeruli were isolated to measure production of PGE, 6-keto-$PGF_{1\alpha}$ and TXB_2; the latter two are stable metabolites of PGI_2 and TXA_2, respectively. Thoracic aortic

TABLE 1

Fatty Acid Composition of the Dietary Lipids

Fatty acid	BT	FO	EPO	EPO:FO
14:0	3.4	8.2	—	2.0
16:0	24.9	18.7	6.4	9.2
16:1	2.6	9.4	—	2.4
18:0	18.7	4.3	1.7	2.4
18:1	40.0	14.6	7.5	9.5
18:2n-6	4.5	2.1	74.9	56.6
18:3n-6	—	—	9.0	7.0
18:3n-3	—	2.4	—	0.6
20:4n-6	—	1.8	—	0.4
20:5n-3	—	16.5	—	4.3
22:5n-3	—	2.5	—	0.6
22:6n-3	—	11.1	—	2.9

Diets contained 150 g fat/kg. Numbers represent percentage of the total fatty acid content.

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Abbreviations: BSA, bovine serum albumin; BT, beef tallow; EBSS, Earls Balanced Salt Solution; EPO, evening primrose oil; FO, fish oil; HDL, high density lipoprotein; PG, prostaglandin; PUFA, polyunsaturated fatty acid; SBO, soybean oil; TXA_2, thromboxane A_2; (V)LDL, (very) low density lipoprotein; GC, gas chromatography; HPLC, high-performance liquid chromatography; PAS, periodic acid-Schiff; RIA, radioimmunoassay.

strips, 5–20 mg dry weight, were incubated and the media assayed for 6-keto-$PGF_{1\alpha}$ production.

Kidney phospholipid fatty acid composition. For this analysis, tissue samples were selected randomly from each dietary group. Kidney tissue was homogenized in saline and extracted with chloroform/methanol (3). Total lipids were separated using a silica SEP PAK cartridge (Waters, Milford, MA); the phospholipid fraction eluted with methanol (4). Phospholipids were methylated in boron trichloride in methanol at 90°C for 1 hr under a stream of nitrogen. Methyl esters of fatty acids were separated on a Supelcowax 10 wide-bore capillary column, using prepurified helium at 12 ml/min as the carrier gas. A 5790A Hewlett-Packard gas chromatograph was used with an injection temperature of 210°C. The GC was interfaced with a 3390A Hewlett-Packard integrator. Fatty acids were identified with a combination of PUFA 1 and PUFA 2 standards (Supelco, Bellefonte, PA) and #68A standard, along with 18:3, 24:0 and 24:1 (NuChek Prep., Elysian, MN). All reagents were high-pressure liquid chromatography (HPLC)-grade (Fisher, Cincinnati, OH).

Glomerular eicosanoids. Five rats in each group were selected randomly for this assay. Kidneys were perfused in vivo with Earls Balanced Salt Solution (EBSS) and heparin (2 U/ml). Glomeruli were isolated using a previously reported sieving technique (5); microscopically, they were at least 80% pure and about 95% decapsulated. Glomeruli were incubated in a total volume of 0.5 ml in EBSS, containing 0.1% bovine serum albumin (BSA) (Fraction V, Sigma, St. Louis, MO), pH 7.4, at 37°C for 1 hr. Samples were then centrifuged immediately, and supernatants saved for eicosanoid radioimmunoassay (RIA) (6). Glomerular pellets were saved for protein determination by the Lowry method (7) after solubilization in 1 M NaOH. For RIA, tritiated eicosanoids and scintillation cocktails were purchased from New England Nuclear (Boston, MA), and standards and antibodies were obtained from Seragen, Inc. (Boston, MA). The smallest detectable doses of eicosanoid standard on the linear portion of the standard curves wre 4.7 pg for PGE, 12.0 pg for 6-keto-$PGF_{1\alpha}$ and 5.2 pg for TXB_2. Nonspecific binding was always less than 3.0%. Intra- and interassay coefficients of variation were 2.7% and 5.5%, respectively, for all assays.

Aortic 6-keto-$PGF_{1\alpha}$ production was measured in the incubation media by RIA after segments of aorta were incubated in EBSS and 0.1% BSA (pH 7.4) at 37°C for 1 hr in a total volume of 1.0 ml. Tissue was air-dried and weighed, and the results wre expressed as ng/mg tissue.

Platelet TXA_2 production was measured after clotting whole blood at 37°C for 30 min with subsequent centrifugation. TXB_2, the stable metabolite of TXA_2, was measured in serum by RIA.

Plasma lipids. HDL cholesterol was measured after precipitation with dextran sulfate-magnesium chloride (Abbott, South Pasadena, CA). Plasma triglycerides, HDL and total cholesterol were measured by enzymatic methods on an ABA100 Bichromatic Analyzer (Abbott Labs.).

Urinary protein was measured using the Bradford reagent (Bio Rad, Rockville Center, NY) and plasma creatinine was determined colorimetrically using the Jaffe reaction.

Light microscopy. Kidney tissue was fixed in Mossman's solution, stained with periodic acid-Schiff (PAS), and coded sections were examined "blinded." In each rat, between 50 and 250 glomeruli were studied. In the glomeruli, particular attention was paid to enlargement with endocapillary swelling, to hyalinosis (capillary protein insulation) and to segmental and global sclerosis. The number of glomeruli with each abnormality was counted, and the results expressed as percentages of total glomeruli studied. The tubular changes evaluated semiquantitatively (0–4+) were dilatation, intraluminal protein, protein reabsorption droplets and nephrocalcinosis.

Statistics. The Kolmogorov-Smirnov test was applied to groups of data to assess whether they were normally or log-normally distributed. For log-normally distributed data, statistical analysis was performed in logarithmically transformed values. Comparisons among groups were tested by one-way ANOVA. Differences between individual groups were established by the Duncan's test.

RESULTS

Weight, blood pressure and renal function (Table 2). In spite of pair-feeding, there were some differences in weight. The lowest weight was in the BT group; weights were significantly higher in each of the FO and EPO, but not in the EPO:FO groups. The mean blood pressures of rats in all groups were normal and did not differ among groups. All rats had nephrotic range proteinuria, with no differences among groups. Mean plasma creatinine in the oil mixture group (EPO:FO) was significantly lower than that of the BT group; but, that of rats in groups fed either FO alone or EPO alone were not different.

Plasma lipids. The results of plasma triglycerides, total cholesterol and HDL cholesterol are shown in Table 3. In the FO, EPO and EPO:FO groups, plasma triglycerides were reduced equally to ca. 50% of that of the BT group. Total cholesterol was reduced by 47% and 20% in the FO and EPO:FO groups, respectively, when compared with BT animals. No difference was observed in the EPO group. Compared with the BT group, HDL levels were significantly increased in the EPO:FO group, but not in the FO or EPO groups.

Fatty acids in kidney phospholipids. The kidney phospholipid fatty acid composition was clearly different in each dietary group (Table 4). The most striking change was in 18:2n-6, which was increased in the FO group, and greatly increased in EPO and EPO:FO groups. There was a marked reduction of 20:4n-6 in the FO group, and a significant but less marked reduction in the EPO:FO group. There was significant incorporation of 20:5n-3 and 22:6n-3 into the FO group. In the EPO:FO group, 22:6n-3 was significantly incorporated, but only small amounts of 20:5n-3 were detectable in 5 of 9 rats. In the groups fed the diets containing EPO, only very small amounts (<1%) of 18:3 n-6 and 20:3 n-6 were detected, indicating a poor incorporation of gammalinolenic acid, although the incorporation of linoleic acid was good.

Eicosanoid production (Table 5). In isolated glomeruli from rats in the FO group, there was a very significant reduction of PGE, 6-keto-$PGF_{1\alpha}$ and TXB_2 production. There was a less marked reduction in these eicosanoids in the EPO:FO group. In the EPO group, TXB_2 production was reduced to about 50% of the BT values.

In aorta, there was a marked reduction in 6-keto-$PGF_{1\alpha}$ production in the FO group, when compared with

TABLE 2

Weight, Blood Pressure and Renal Function

	BT (n = 10)	FO (n = 10)	EPO (n = 10)	EPO:FO (n = 11)
Weight[a] (g) wk 5	244 ± 17	263 ± 15[c]	258 ± 11[c]	254 ± 11
Blood pressure[a] (mm Hg) wk 5	111 ± 11	112 ± 7	111 ± 8	116 ± 6
Urine protein excretion[b] (mg/23 h) wk 4	368 (266, 509)	305 (247, 377)	317 (214, 468)	374 (246, 568)
Plasma creatinine[b] (mg/dl) wk 6	0.76 (0.60, 0.97)	0.64 (0.53, 0.77)	0.68 (0.51, 0.92)	0.55[c] (0.43, 0.72)

[a]Values are means ±1 SD.

[b]Values are geometric means (−1 SD, +1 SD).

[c]$p < 0.05$ vs BT.

TABLE 3

Plasma Triglycerides, Cholesterol and HDL Cholesterol

	BT (n = 10)	FO (n = 10)	EPO (n = 10)	EPO:FO (n = 11)
Triglycerides (mg/dl)	801 (479, 1341)	362[a] (282, 463)	379[a] (269, 535)	404[a] (304, 537)
Total cholesterol (mg/dl)	764 (647, 902)	409[a] (336, 499)	797[b] (650, 977)	622[a,b,c] (463, 835)
HDL cholesterol (mg/dl)	179 (95, 338)	151 (126, 182)	197 (151, 258)	259[a,b] (243, 318)

Values are geometric means (−1 SD, +1 SD).

[a]$p < 0.05$ vs BT.

[b]$p < 0.05$ vs FO.

[c]$p < 0.05$ vs EPO.

TABLE 4

Fatty Acids in Kidney Phospholipids

	BT (n = 8)	FO (n = 10)	EPO (n = 9)	EPO:FO (n = 9)
16:0	20.9 ± 6.9	20.7 ± 6.2	17.9 ± 2.0	20.2 ± 5.4
16:1	1.2 ± 0.2	2.6 ± 0.3[a]	0.7 ± 0.3[b]	1.6 ± 1.2[b,c]
18:0	24.0 ± 2.7	21.4 ± 2.6[a]	25.6 ± 1.9[b]	24.1 ± 2.5[b]
18:1	16.2 ± 1.0	12.5 ± 1.0[a]	9.2 ± 0.7[a,b]	11.4 ± 3.2[a,c]
18:2n-6	5.0 ± 0.8	7.2 ± 1.0[a]	12.9 ± 1.1[a,b]	14.9 ± 1.4[a,b,c]
20:4n-6	29.2 ± 7.5	16.5 ± 3.0[a]	30.9 ± 4.1[b]	23.9 ± 3.6[a,b,c]
20:5n-3	—	11.8 ± 2.8	—	1.7 ± 0.6*
22:6n-3	2.7 ± 0.9	6.6 ± 0.5[a]	1.8 ± 0.7[a,b]	3.8 ± 0.7[a,b,c]

Values are percentage of major GC peaks ± SD.

[a]$p < 0.05$ vs BT.

[b]$p < 0.05$ vs FO.

[c]$p < 0.05$ vs EPO.

*Detectable levels of 20:5n-3 were present in only 5 rats in group EPO:FO.

TABLE 5

Eicosanoid Production by Isolated Glomeruli, Aorta and Platelets

	BT	FO	EPO	EPO:FO	Number of observations
Isolated glomeruli					
PGE (ng/mg protein)	6.7 (3.8, 11.9)	0.2[a] (0.1, 0.3)	7.4[b] (5.9, 9.2)	0.9[a,b,c] (0.5, 1.5)	4,5,5,6
6-Keto-$PGF_{1\alpha}$ (ng/mg protein)	4.3 (2.8, 6.9)	0.1[a] (0.07, 0.11)	2.7[b] (2.3, 3.3)	0.4[a,b,c] (0.25, 0.73)	3,5,5,5
TXB_2 (ng/mg protein)	5.6 (3.8, 8.1)	0.3[a] (0.2, 0.4)	2.8[a,b] (2.0, 4.0)	1.2[a,b,c] (0.8, 1.7)	4,5,5,5
Aorta					
6-Keto-$PGF_{1\alpha}$ (ng/mg tissue)	43.9 (24.2, 79.7)	4.3[a] (2.2, 8.5)	52.0[b] (33.4, 81.1)	30.1[b,c] (20.6, 44.0)	10,9,10,11
Platelets					
TXB_2 (ng/ml serum)	145.2 (85.5, 246.7)	45.6[a] (29.3, 71.2)	145.0[b] (72.0, 292.2)	101.1[b] (58.3, 175.3)	10,10,10,9

Values are geometric means (−SD, +SD).

[a] $p < 0.05$ vs BT.

[b] $p < 0.05$ vs FO.

[c] $p < 0.05$ vs EPO.

all other groups. In the EPO:FO group, 6-keto-$PGF_{1\alpha}$ production was not significantly different than that of the BT group, although it was lower than that of the EPO group.

Platelet TXB_2 production was significantly lower only in rats in the FO group compared with all other groups. No significant difference was found among EPO, EPO:FO and BT groups.

Light microscopy. There were minor to moderate tubular changes of dilatation and intraluminal protein, but no differences were found among groups. No glomerular alterations were observed in any of the rats.

DISCUSSION

The primary purpose of this experiment was to study the value, compared with saturated fat, of dietary n-6, n-3 PUFA and a mixture of both, on various lipid classes in rat plasma, kidney, aorta and platelets. We hoped that the combination of n-6 and n-3 would eliminate the disadvantages of n-3 PUFA, but preserve the beneficial effects. The dose of adriamycin used induced a slowly progressive renal disease permitting assessment of early lipid changes. In previous experiments (1,2), a dose of 7.5 mg/kg adriamycin induced a very severe nephrotic syndrome, with renal failure, marked renal histologic abnormalities and extreme increases in plasma cholesterol and triglycerides. Adriamycin given at 3 mg/kg induced a fully developed nephrotic syndrome, but renal function and histology were largely preserved and the elevations of plasma lipids were not as marked. This may also explain why, in the present experiment, the diets containing FO did not have an effect on plasma creatinine, because the BT group had a mildly elevated creatinine (0.76 mg/dl), and in our previous report using the adriamycin dose of 7.5 mg/kg, the mean plasma creatinine of the BT group was 0.98 mg/dl (2). Differences in renal function and blood pressure among dietary groups were not anticipated and were not found. We did see a significant decrease in plasma creatinine in the EPO:FO group, suggesting that the combination diet may best preserve renal function, compared with the FO and EPO diets, even at an early stage. The differences in creatinine cannot be explained by differences in weight (Table 2). A long-term experiment with serial measurement of renal function would help to clarify the potential importance of these differences in plasma creatinine. The FO and EPO groups had significantly higher weights than the BT group, in spite of the pair-feedings. The possible influence of these differences on the variables studied is unknown.

The combination of EPO and FO (75:25) lowered plasma triglycerides by 50%, compared with the BT group, and was not different from that of FO or EPO alone. n-6 PUFA have been reported to have beneficial effects on the hypertriglyceridemia of patients with Type IIb hyperlipidemia (8) and patients with chronic renal failure (9). n-3 PUFA feedings reduce plasma total triglycerides, low and very low density lipoprotein (LDL and VLDL) levels in normal (10) and hyperlipidemic patients (8). In normal rats, fish oil reduces plasma triglycerides, but not cholesterol (11); in nephrotic rats (1), fish oil reduces serum triglycerides, total cholesterol and LDL cholesterol. Many investigators attribute the hypolipidemic effect of fish oil in humans to reduction of VLDL triacylglycerol (12), and VLDL apoprotein-B synthesis in the liver (13), leading to a reduction in LDL formation.

The combination of EPO and FO significantly lowered plasma total cholesterol, although not as markedly as FO. Unexpectedly, EPO alone did not have a significant effect on total cholesterol, although the hypocholesterolemic effect of n-6 PUFA has been reported in some human studies (10,12). The rise in HDL in the EPO:FO group was unexpected in view of the lack of effect observed in the FO and EPO groups. In humans with Type

II hyperlipidemia, those who were fed vegetable oil rich in n-6 PUFA had a small but significant increase in plasma HDL (8). The HDL:cholesterol ratio increased in chronic renal failure patients during n-6 PUFA feeding (9). Literature on the effects of fish oil on HDL levels report variable results. The depression of plasma-HDL levels by a fish-oil diet has been documented in humans (8,12,13) and monkeys (14), although others report no change in humans (10,15) or rats (1). Conversely, other investigators report an increase in HDL in patients with chronic renal failure who were fed a fish oil diet with a higher HDL_2:HDL_3 ratio (16). In a study of patients having angina, hyperlipidemia or previous myocardial infarctions (17) and chronic ambulatory peritoneal dialysis patients (18), those fed fish oil showed increased HDL_2 and total HDL. Because HDL is thought to be protective against atherosclerosis, the effect on HDL by the EPO:FO combination is potentially important. A more detailed study of the lipoprotein classes and apoprotein levels is clearly indicated.

There was good incorporation of most of the dietary PUFA into kidney phospholipids. Compared with the BT group, 18:2n-6 levels increased in all three diets, but were the highest in the EPO:FO group. In the groups fed the diets containing EPO, the incorporation of 18:3n-6 was very small, suggesting that the biological effects of the EPO were due mainly to its very high content of 18:2n-6. 20:5n-3 and 22:6n-3 were increased and 20:4n-6 was reduced in the FO and EPO:FO groups, compared with the BT and EPO groups. Our data are in agreement with studies in normal rats (20) and also exemplify the interaction of various PUFA modifying their rates of desaturation, chain elongation and esterification (21) in phospholipids and neutral lipids. What the contributions of n-3 and n-6 PUFAs to this model are speculative. PUFA substitution in phospholipids presumably would have a direct effect on cell membrane fluidity and lipoprotein structure. These may, in turn, affect receptor response to lipoproteins and prostaglandin synthesis upon stimulation.

A potentially beneficial effect of the combination diet was the preservation of aortic synthesis of PGI_2. The FO diet markedly inhibited PGI_2, yet the EPO:FO and EPO diets maintained PGI_2 at levels not significantly different than those of the BT group. The eicosanoid data in isolated glomeruli showed decreased levels of TXB_2 in EPO, FO and EPO:FO groups, and decreased levels of 6-keto-$PGF_{1\alpha}$ and PGE in the FO and EPO:FO groups. Both aortic 6-keto-$PGF_{1\alpha}$ and platelet TXB_2 levels in the rats fed the EPO:FO diet were ca. 70% of those from rats fed EPO, suggesting that FO added to the diet suppresses the synthesis of PG and TX to the same extent. The magnitude of the suppression probably depends on the relative amounts of n-3 and n-6 PUFA in the diets. It would be desirable to formulate a diet that preserves the vasodilatory effects of PGE and PGI_2, while decreasing the vasoconstriction and platelet aggregatory effects of TXA_2. In the nephrotic rat, we believe that a combination of EPO:FO may contribute to preservation of renal function. We have previously reported the protective effects of PUFA on the progression of renal disease in the rat with nephrotic syndrome induced by adriamycin 7.5 mg/kg (1,2) and in other nonimmunologic models of renal disease (22,23). Glomerulosclerosis may be the result of similar pathogenic events to those that lead to atherosclerosis (24). In the model of chronic aminonucleoside nephrosis in the rat, glomerulosclerosis is accelerated by dietary cholesterol supplementation (25). The reduction of atherosclerotic risk factors could also prevent long-term renal deterioration. The combination of n-6 and n-3 PUFA needs to be tested further in models with progressive chronic renal disease.

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REFERENCES

1. Ito, Y., Barcelli, U., Yamashita, W., Weiss, M., Thompson, B., Kashyap, M., Deddens, J., and Pollak, V.E. (1987) *Life Sci. 40,* 2317-2324.
2. Ito, Y., Barcelli, U., Yamashita, W., Weiss, M., Kashyap, M., Glas-Greenwalt, P., and Pollak, V.E. (1988) *Metabolism 37,* 352-357.
3. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) *J. Biochem. Chem. 226,* 497-509.
4. Juaneda, P., and Rocquelin, G. (1985) *Lipids 20,* 40-41.
5. Hassid, A., Konieczkowski, M., and Dunn, M.J. (1979) *Proc. Natl. Acad. Sci. USA 76,* 1155-1159.
6. Gallon, L.S., and Barcelli, U.O. (1986) *Prostaglandins 31,* 217-225.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) *J. Biol. Chem. 193,* 265-277.
8. Phillipson, B.E., Rothrock, D.W., Connor, W.E., Harris, W.S., and Illingworth, D.R. (1985) *N. Engl. J. Med. 312,* 1210-1216.
9. Tsukamoto, Y., Okubo, M., Tomiko, Y., Marumo, F., and Nakamura, H. (1982) *Nephron 31,* 236-241.
10. Harris, W.S., Connor, W.E., and McMurry, M.P. (1983) *Metabolism 32,* 179-184.
11. Wong, S.H., Nestel, P.J., Trimble, R.P., Storer, G.B., Illman, R.J., and Topping, D.L. (1984) *Biochim. Biophys. Acta 792,* 103-109.
12. Nestel, P.J., Connor, W.E., Reardon, M.F., Connor, S., Wong, S., and Boston, R. (1984) *J. Clin. Invest. 74,* 82-89.
13. Illingworth, D.R., Harris, W.S., and Connor, W.E. (1984) *Arteriosclerosis 4,* 270-275.
14. Davis, H.R., Bridenstine, R.T., Vesselinovitch, D., and Wissler, R.W. (1987) *Arteriosclerosis 7,* 441-449.
15. Sanders, T.A.B., Sullivan, D.R., Reeve, J., and Thompson, G.R. (1985) *Arteriosclerosis 5,* 459-465.
16. Rylance, P.B., Gordge, M.P., Saynor, R., Parsons, V., and Weston, M.J. (1986) *Nephron 43,* 196-202.
17. Saynor, R., Verel, D., and Gillott, T. (1984) *Atherosclerosis 50,* 3-10.
18. van Acker, B.A.C., Bilo, H.J.G., Popp-Snijders, C., van Bronswijk, H., Oe, P.L., and Donker, A.J.M. (1987) *Nephrol. Dial. Transplant. 2,* 557-561.
19. Sanders, T.A.B., and Hochland, M.C. (1983) *Br. J. Nutr. 50,* 521-529.
20. Huang, Y.S., Nassar, B.A., and Horrobin, D.F. (1986) *Biochim. Biophys. Acta 879,* 22-27.
21. Cook, H.W., and Spence, M.W. (1987) *Lipids 22,* 613-619.
22. Barcelli, U.O., Weiss, M., and Pollak, V.E. (1982) *J. Lab. Clin. Med. 100,* 786-797.
23. Barcelli, U., Miyata, J., Ito, Y., Gallon, L., Laskarzewski, P., Weiss, M., Hitzemann, R., and Pollak, V. (1986) *Prostaglandins 32,* 211-219.
24. Diamond, J.R., and Karnovsky, M.J. (1988) *Kidney Int. 33,* 917-924.
25. Diamond, J.R., and Karnovsky, M.J. (1987) *Kidney Int. 32,* 671-677.

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The Composition of Red Cell Membrane Phospholipids in Canadian Inuit Consuming a Diet High in Marine Mammals

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A study of the fatty acid composition of red cell phosphatidylcholine and phosphatidylethanolamine and serum cholesterol was undertaken in 185 Canadian Inuit (age 2 months–82 years). Samples from 24 Canadian men and women (21–50 years) living in Vancouver were also analyzed as a reference for the Inuit in this age range. Dietary survey of the Inuit community (325 Inuit) demonstrated a diet based on traditional foods in which the principal source of n-3 fatty acid was marine mammal flesh (mean intake: 164 g/person/day) rather than fish (mean intake: 13 g/person/day). Compared to the Vancouver samples, the Inuit phosphatidylethanolamine had higher 20:5n-3 and 22:6n-3 and lower 20:4n-6, but similar 18:2n-6 levels. The level of 20:5n-3 was higher and 20:4n-6 was lower in the Inuit than in the Vancouver red cell phosphatidylcholine. Despite these differences in percentage content of C20 and C22 n-6 and n-3 fatty acids, the mean chain length and unsaturation index of the Inuit and Vancouver red cell phosphatidylcholine and phosphatidylethanolamine were very similar. Serum cholesterol concentration showed no sex difference within the Inuit, and no difference from Vancouver men and women of similar age. The analyses suggest that the fatty acid composition of the Inuit red cell phospholipids are primarily a reflection of their diet-fat composition.

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Considerable interest has been focused on the n-3 fatty acids, eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), since the studies by Bang et al. showed a relationship in Greenland Eskimo between a high dietary intake of n-3 fatty acids and a disease pattern characterized by a particularly low incidence of ischemic heart disease (1–5). Clinical studies utilizing fish-oil concentrates, or addition of fatty fish to Western diets, have since suggested that the beneficial effect of n-3 fatty acids may involve several mechanisms including altered platelet function and platelet and vessel wall eicosanoid biosynthesis (6–8), as well as increased red cell deformability and reduced blood viscosity related to the incorporation of dietary C20 and C22 n-3 fatty acids into the red cell membrane (9–11). Despite these biochemical changes in trials with fish oils, the efficacy of dietary fish in reducing mortality from ischemic heart disease is uncertain as both a reduction (12,13) and no reduction (14–16) in disease incidence has been reported in free-living populations consuming fish-rich diets.

During the early 1970s, the major portion of the fat in the Eskimo diet was from marine mammals, rather than from fish (2,4). Although marine mammal fat contains high levels of n-3 fatty acids, the relative quantities of 20:5n-3, 22:5n-3 and 22:6n-3 (17) differ from that in fish (18,19). Further, the preferential acylation of n-3 fatty acids to the glycerol *sn*-2 position in fish rather than to *sn*-1 and *sn*-3 in the triacylglycerols of marine mammals has recently been emphasized, and may be expected to lead to potentially important differences in absorption and metabolism of n-3 fatty acids from these two diet lipid sources (20).

In September 1985, a dietary survey with collection of blood samples, for serum assays not related to fatty acids, was conducted in the Canadian Arctic community of Broughton Island, Northwest Territories. This island, which lies off the East coast of Baffin Island, has ca. 400 residents and was selected for study because previous hunting and fishing surveys indicated a high-potential per capita consumption of native foods, marine foods are abundantly available and the high cost of air transportation continues to be a barrier to the extensive use of other foods. Red cell fatty acids are known to reflect diet fat composition (21,22) and, through modulation of membrane properties, may contribute to the low incidence of ischemic heart disease (9–11) in Eskimo (1,3). Thus, the opportunity was taken to analyze the fatty acid composition of red cell phospholipids (PL) of Broughton Island Inuit in order to document the composition of these cell membranes in a population that continues to consume a largely traditional diet high in marine mammals.

METHODS

Dietary surveys were conducted by direct interview in the native language (Inuktitut) to collect data on patterns of traditional food usage and quantitative food intake data for each household. A summary of the per capita intake of native foods for 325 Inuit who participated in the diet survey is given in Table 1. Further details on the survey are not part of this report, but can be obtained from author HK. The fatty acid composition of marine mammals caught by the Inuit hunters during the survey has been reported (17).

Blood sampling. A venous blood sample was drawn into Venoject (Terume Med. Corp., Elkton, MD) serum separation tubes. The tubes were centrifuged and all but 100–200 μl serum removed for other analyses. The tubes containing the red cell pellet were immediately frozen and shipped to Vancouver where they were transferred to storage at −80°C until analyzed. Samples from 185 Inuit were received and grouped by age and sex for subjects over 11 yrs; children of both sexes were grouped together, 0–5 or 6–10 yrs (Table 2). For comparative purposes, blood samples from 12 men and 12 women consuming a mixed diet and working in the Research Centre in Vancouver were drawn, stored and processed to resemble the handling of the Inuit samples as closely as practically possible.

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; GLC, gas liquid chromatography; TLC, thin layer chromatography; UI, unsaturation index.

TABLE 1

Profile of Native Food Intake on Broughton Island (mean for 312 people)

	g/person/day
Crowberries	89
Ringed seal meat	80
Caribou meat	31
Narwhal meat	29
Narwhal matak	15
Char meat	13
Walrus meat	12
Ringed seal blubber	8
Kelp	7
Bearded seal meat	6
Blueberries	4
Ringed seal liver	3
Narwhal blubber	3
Walrus blubber	3
Mussels	3
Bearded seal, other parts	2
Walrus matak	2
Bearded seal blubber	1
Caribou fat	1
Eider duck	<1
Ringed seal, other parts	<1
Beluga meat	<1
Total marine mammal	164
Total	13

TABLE 2

Number of Subjects Studied and Sample Distribution by Age and Sex

	Inuit		Vancouver	
Age yrs	Male n	Female n	Male n	Female n
0-5	6	7		
6-10	4	5		
11-15	11	17		
16-20	9	12		
21-50	41	59	12	12
>50	14	13		

Red blood cell and plasma cholesterol analyses. Immediately upon removal from frozen storage, the glass tubes containing the red cells were broken and 1 cm^2 of cells was sliced from the bottom of each pellet. The cells were lysed (21), the membranes pelleted, washed three times and the lipids extracted (22,23). The PL were separated from other lipid classes (24), then further resolved into their individual PL classes (25) by thin layer chromatography (TLC) identified on the basis of R_f by cochromatography of known standards. The bands corresponding to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were recovered, the fatty acid components converted to their respective methyl esters with methanolic HCl and analyzed by capillary-column gas liquid chromatography (GLC). Details of the GLC and data handling have been reported earlier (17). The unsaturation index (UI) and mean chain length of the fatty acids were calculated as described previously (24).

Total cholesterol was assayed in 10 μl of serum using an enzymatic kit (Biopacific diagnostics Inc., North Vancouver, British Columbia).

RESULTS AND DISCUSSION

The dietary survey conducted during September 1985 confirmed a high intake of traditional foods by the Broughton Island Inuit. The average intake of fish (char) was ca. 13 g, but the intake of total marine mammal tissue was ca. 164 g per person per day (Table 1). The extensive use of marine mammals in the diet of these Inuit is similar to the higher intake of seal and whale (higher than fish) by the Greenland Eskimo during the Spring of 1970 (2,4).

The fatty acid composition of PE and PC from the Inuit and Vancouver PE and PC is shown in Tables 3 and 4, respectively. The Vancouver reference data is similar to previous reports for apparently healthy adults consuming mixed Western diets (26,27). The data for the Inuit red cells demonstrate the incorporation of dietary C20 and C22 n-3 fatty acids in all age groups into the membrane PE and PC, and the higher level of these fatty acids in PE than PC. No differences were found in the composition of PE between male and female Inuit (Table 3); PC was also similar between the sexes, except for a higher 20:0 in the 11-15 yr old girls than in the boys (Table 4).

When compared with Vancouver residents of similar age (21-50 yrs), the Inuit had a higher n-3 series 18:3, 18:4, 20:5 and 22:6, and a lower n-6 series 20:4, 22:4 and 22:5 in the red cell PE (Table 3). The most pronounced difference in the n-6 series was in 22:4n-6; the level in the Inuit was ca. 27% of the amount in the Vancouver samples. The total C20 plus C22 n-6 and n-3 fatty acids were lower and higher, respectively in the Inuit; however, the UI and the mean chain length remained similar to the Vancouver samples.

The analyses of PC from the Inuit males showed lower n-6 series 20:3 and 22:5 and higher n-3 series 18:3, 18:4, 20:5 and 22:6 than in Vancouver males (Table 4). The Inuit females had lower n-6 series 18:2 and 20:3 and higher n-3 series 18:3, 18:4 and 20:5 than their Vancouver counterparts. The apparently higher 22:6n-3 in Inuit is explained by the low 22:6n-3 in the Vancouver group. The reason for this is unknown. The total C20 and C22 n-6 and n-3 fatty acids, UI and mean chain length of PC was similar between the Inuit and Vancouver groups, except for a higher C20 plus C22 n-3 series in the Inuit males. The latter was also due to the low 22:6n-3 in the Vancouver men.

The levels of 20:5n-3 and 22:6n-3 in the Inuit PE in this study are higher than those found following supplementation with fish oil concentrates, e.g., containing ca. 19%, 3%, 12% or 31%, 6% and 19% 20:5n-3, 22:5n-3, 22:6n-3, respectively (9,11). The seal and whale caught by the Inuit during the study contained 9-12%, 20:5n-3, 5-6% 22:5n-3, 9-10% 22:6n-3 (17), indicating that, like human red cell PL (26,27, Tables 3 and 4), these mammals contain less 20:5n-3, particularly when related to the content of 22:5n-3 and 22:6n-3, than fish (18,19). The effect of these differences in fatty acid composition, and in the triglyceride structure between mammals and fish (20), on tissue fatty acid composition has not been studied.

TABLE 3

Major Fatty Acid of Erythrocyte Phosphatidylethanolamine

Fatty acids % total	Inuit										Vancouver	
	Children		Males				Females				Male	Female
	0-5	6-10	11-15	16-20	21-50	>50	11-15	16-20	>50	21-50	21-50	21-50
Saturates												
14:0	0.8 ± 0.2	1.0 ± 0.3	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.9 ± 0.2	0.1 ± 0.1	0.2 ± 0.1
16:0	17.8 ± 5.8	15.5 ± 3.4	13.6 ± 1.1	11.2 ± 0.2	12.0 ± 0.8†	15.0 ± 0.9	19.0 ± 6.2	12.4 ± 0.7	13.1 ± 1.0†	15.0 ± 0.8	8.8 ± 0.8	8.1 ± 0.6
18:0	16.2 ± 0.9	12.6 ± 2.6	11.4 ± 0.8	12.9 ± 0.1	11.2 ± 0.5†	11.1 ± 0.7	11.6 ± 1.4	12.0 ± 1.2	11.9 ± 0.9	9.3 ± 0.9	13.6 ± 0.2	11.9 ± 0.3
20:0	0.6 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.0
Monoenes												
16:1	2.0 ± 0.7	3.2 ± 1.3	1.2 ± 0.4	0.8 ± 0.4	1.4 ± 0.2	2.6 ± 0.9	1.4 ± 0.4	1.1 ± 0.4	1.3 ± 0.2	3.1 ± 0.9	1.2 ± 0.2	1.0 ± 0.1
18:1	20.5 ± 1.2	23.4 ± 2.1	21.3 ± 1.1	22.7 ± 1.1	22.3 ± 0.6†	21.7 ± 2.8	19.3 ± 1.7	22.1 ± 0.7	20.9 ± 0.6	20.0 ± 0.4	15.8 ± 1.0	18.6 ± 1.0
20:1	0.8 ± 0.2	1.0 ± 0.4	0.4 ± 0.1	0.5 ± 0.3	0.4 ± 0.1	0.5 ± 0.2	0.6 ± 0.1	0.7 ± 0.3	0.5 ± 0.2	0.8 ± 0.3	0.5 ± 0.1	0.6 ± 0.2
22:1	0.3 ± 0.1	0.4 ± 0.2	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.2	1.2 ± 1.1	0.1 ± 0.0	0.1 ± 0.0
n-6												
18:2	8.0 ± 1.9	8.0 ± 1.2	8.3 ± 0.9	8.2 ± 1.3	6.8 ± 0.4	5.3 ± 0.6	7.3 ± 0.5	8.4 ± 0.6	5.6 ± 0.3	5.6 ± 0.9	6.8 ± 0.4	6.8 ± 1.0
18:3	0.8 ± 0.6	0.5 ± 0.3	0.4 ± 0.1	0.2 ± 0.2	0.2 ± 0.1†	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.8 ± 0.1	0.4 ± 0.3
20:3	3.1 ± 1.3	2.1 ± 0.5	1.4 ± 0.5	0.6 ± 0.3	0.3 ± 0.1†	0.4 ± 0.1	1.2 ± 0.4	1.2 ± 0.3	0.8 ± 0.2	0.4 ± 0.2	1.1 ± 0.1	1.0 ± 0.1
20:4	12.7 ± 1.1	15.0 ± 2.8	17.5 ± 1.4	19.2 ± 1.1	15.1 ± 1.0†	12.0 ± 0.9	15.2 ± 2.3	18.6 ± 1.1	15.3 ± 1.2†	13.4 ± 1.6	25.7 ± 0.6	25.8 ± 1.5
22:4	2.2 ± 0.6	3.4 ± 1.4	2.8 ± 0.2	3.8 ± 0.1	1.9 ± 0.2†	1.3 ± 0.1	2.4 ± 0.3	3.1 ± 0.3	2.1 ± 0.2†	1.5 ± 0.2	7.0 ± 0.7	7.6 ± 1.5
22:5	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.0	0.6 ± 0.2	0.4 ± 0.1†	0.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.3	0.3 ± 0.0†	0.2 ± 0.0	0.9 ± 0.0	0.8 ± 0.2
n-3												
18:3	1.2 ± 0.9	0.8 ± 0.5	0.9 ± 0.3	1.0 ± 0.6	1.4 ± 0.2†	1.3 ± 0.3	1.1 ± 0.2	0.8 ± 0.3	1.4 ± 0.2†	0.8 ± 0.4	0.3 ± 0.1	0.2 ± 0.1
18:4	0.0 ± 0.0	0.4 ± 0.2	1.1 ± 0.6	0.5 ± 0.3	0.7 ± 0.2†	0.9 ± 0.3	0.7 ± 0.2	0.9 ± 0.4	0.8 ± 0.3†	1.5 ± 0.9	0.1 ± 0.0	0.1 ± 0.0
20:5	2.2 ± 0.6	2.0 ± 0.6	3.6 ± 1.0	3.6 ± 1.6	7.1 ± 0.6†	9.6 ± 1.2	3.0 ± 0.4	3.4 ± 0.7	7.5 ± 0.9†	9.9 ± 1.4	1.6 ± 0.1	1.7 ± 0.4
22:5	3.1 ± 0.8	3.2 ± 0.5	5.0 ± 0.4	4.5 ± 0.9	5.2 ± 0.2	5.4 ± 0.5	3.9 ± 0.5	4.3 ± 0.4	5.5 ± 0.4	4.8 ± 0.5	5.5 ± 0.6	6.6 ± 0.7
22:6	5.7 ± 1.7	5.7 ± 1.0	9.0 ± 0.6	7.9 ± 2.2	11.3 ± 1.7†	10.0 ± 1.0	9.5 ± 1.4	7.7 ± 0.8	10.2 ± 0.7†	9.4 ± 0.2	7.4 ± 0.2	7.2 ± 1.5
ΣC≥20ω-6	19.7 ± 4.2	21.4 ± 5.0	22.3 ± 1.3	24.4 ± 0.9	18.6 ± 1.3†	14.6 ± 0.8†	20.4 ± 2.8	23.9 ± 1.7	18.8 ± 1.6	15.9 ± 1.6	36.9 ± 1.0	35.9 ± 2.7
ΣC≥20ω-3	11.1 ± 3.0	11.0 ± 2.0	18.2 ± 1.8	16.0 ± 4.7	23.6 ± 2.1†	24.9 ± 2.6†	17.7 ± 1.7	15.4 ± 1.8	23.2 ± 1.9	24.9 ± 3.3	14.8 ± 0.3	15.3 ± 2.5
X chain Lth	18.5 ± 0.4	18.6 ± 0.3	18.9 ± 0.0	18.9 ± 0.1	18.9 ± 0.1	18.8 ± 0.1	18.7 ± 0.2	18.9 ± 0.0	19.0 ± 0.1	18.8 ± 0.2	19.3 ± 0.1	19.3 ± 0.0
UI	182 ± 30	189 ± 22	232 ± 8	232 ± 12	214 ± 10	237 ± 14	230 ± 6	230 ± 6	243 ± 7	244 ± 9	250 ± 4	234 ± 7

Data are given as means ± SE by age and sex for Inuit and Vancouver residents (Table 2) for major fatty acids carbon chain length ≥14.

*, † indicate values significantly different $p \leq 0.05, 0.01$, respectively, for Inuit males or females from Vancouver males or females, respectively.

UI, unsaturation index.

TABLE 4

Major Fatty Acids of Erythrocyte Phosphatidylcholine

Fatty acids % total	Inuit										Vancouver	
	Children		Males				Females				Male	Female
	0–5	6–10	11–15	16–20	21–50	>50	11–15	16–20	>50	21–50	21–50	21–50
Saturates												
14:0	0.2 ± 0.1	0.8 ± 0.4	0.4 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	1.3 ± 0.5	1.2 ± 0.2	0.9 ± 0.2	0.1 ± 0.1	0.3 ± 0.1
16:0	22.5 ± 3.0	27.1 ± 2.1	19.5 ± 1.2	17.3 ± 2.2	21.6 ± 1.4	25.4 ± 1.4	19.0 ± 0.8	18.3 ± 2.2	23.0 ± 0.8	21.8 ± 2.6	19.6 ± 2.8	14.4 ± 0.7
18:0	14.2 ± 1.1	14.6 ± 2.6	13.8 ± 0.9	17.5 ± 2.6	15.5 ± 0.8	14.1 ± 1.3	12.3 ± 1.2	13.1 ± 1.2	13.4 ± 0.5	15.2 ± 2.1	15.9 ± 0.5	15.0 ± 0.5
20:0	1.4 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	2.0 ± 0.7	0.8 ± 0.1	0.8 ± 0.2	1.6 ± 0.3	1.8 ± 0.3‡	0.8 ± 0.1	1.3 ± 0.4	0.5 ± 0.1	0.6 ± 0.2
Monoenes												
16:1	1.8 ± 0.5	3.4 ± 1.7	2.0 ± 0.5	1.9 ± 0.3	1.8 ± 0.3	2.8 ± 0.5	3.4 ± 0.7	3.9 ± 1.7	2.9 ± 0.4†	4.2 ± 1.6	2.7 ± 1.1	1.5 ± 0.4
18:1	24.1 ± 1.8	20.5 ± 2.0	23.3 ± 2.0	19.9 ± 1.8	21.0 ± 0.8	22.8 ± 2.1	21.4 ± 1.9	23.3 ± 1.4	20.1 ± 0.8	20.7 ± 2.5	22.5 ± 1.1	23.1 ± 1.0
20:1	0.4 ± 0.0	0.1 ± 0.0	1.4 ± 0.8	0.3 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.7 ± 0.1	0.9 ± 0.3	0.8 ± 0.1	0.4 ± 0.2	1.3 ± 0.3	1.1 ± 0.4
22:1	0.6 ± 0.2	0.2 ± 0.1	0.8 ± 0.4	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.3 ± 0.3
n-6												
18:2	13.9 ± 3.4	17.1 ± 3.6	16.6 ± 0.8	13.8 ± 3.8	13.9 ± 0.8	10.8 ± 1.8	14.7 ± 1.1	14.4 ± 0.9	13.9 ± 0.8†	11.7 ± 2.0	15.7 ± 3.0	18.9 ± 0.3
18:3	0.3 ± 0.1	0.1 ± 0.1	0.8 ± 0.2	0.1 ± 0.1	0.3 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.3	1.3 ± 0.6	0.4 ± 0.2
20:3	0.4 ± 0.1	1.3 ± 0.9	1.0 ± 0.4	0.4 ± 0.1	0.5 ± 0.1†	0.5 ± 0.1	1.0 ± 0.3	0.4 ± 0.2	0.8 ± 0.1†	0.2 ± 0.1	2.3 ± 0.4	2.0 ± 0.4
20:4	9.6 ± 2.9	6.9 ± 1.3	8.8 ± 2.1	12.7 ± 2.6	0.6 ± 1.0	7.4 ± 1.3	10.7 ± 3.1	10.7 ± 2.5	9.9 ± 1.0	9.4 ± 2.1	9.2 ± 1.4	11.3 ± 1.2
22:4	1.2 ± 0.0	1.7 ± 0.7	0.8 ± 0.2	1.1 ± 0.4	1.0 ± 0.2	1.3 ± 0.6	0.3 ± 0.1	1.2 ± 0.4	0.4 ± 0.1	0.8 ± 0.3	1.0 ± 0.6	0.8 ± 0.2
22:5	0.4 ± 0.2	0.2 ± 0.0	0.3 ± 0.1	0.6 ± 0.2	0.4 ± 0.0†	0.2 ± 0.1	0.9 ± 0.3	0.4 ± 0.2	0.8 ± 0.3	0.5 ± 0.2	0.1 ± 0.0	3.3 ± 0.1
n-3												
18:3	1.3 ± 0.4	1.1 ± 0.2	0.8 ± 0.2	1.0 ± 0.1	0.9 ± 0.2†	0.8 ± 0.0	0.9 ± 0.3	0.8 ± 0.3	0.7 ± 0.1†	1.2 ± 0.4	0.0 ± 0.0	0.1 ± 0.1
18:4	2.1 ± 1.5	0.7 ± 0.2	0.8 ± 0.5	2.1 ± 0.7	1.3 ± 0.4†	1.1 ± 0.3	2.5 ± 1.7	1.7 ± 0.6	2.5 ± 0.6†	2.0 ± 1.0	0.2 ± 0.1	0.2 ± 0.1
20:5	0.4 ± 0.1	2.4 ± 1.0	1.5 ± 0.3	1.1 ± 0.2	2.7 ± 0.4†	2.8 ± 0.3	2.2 ± 0.5	1.4 ± 0.1	2.8 ± 0.4*	3.2 ± 0.9	1.0 ± 0.2	1.8 ± 0.3
22:5	0.4 ± 0.0	0.6 ± 0.0	1.2 ± 0.4	1.2 ± 0.3	1.4 ± 0.2	2.1 ± 0.5	0.8 ± 0.1	0.8 ± 0.2	1.5 ± 0.4	1.1 ± 0.3	1.7 ± 0.3	1.8 ± 0.4
22:6	2.5 ± 1.0	1.2 ± 0.3	2.3 ± 0.3	3.5 ± 0.8	3.9 ± 0.4†	3.1 ± 0.6	2.2 ± 0.3	2.2 ± 0.3	3.4 ± 0.4	4.0 ± 1.2	1.6 ± 0.4	3.7 ± 0.6
ΣC≥,ω-6	11.9 ± 2.9	10.4 ± 0.6	12.4 ± 2.4	17.0 ± 3.3	12.7 ± 1.2	10.4 ± 1.9	16.5 ± 3.2	15.0 ± 3.4	13.6 ± 1.2	11.6 ± 3.7	12.7 ± 2.6	14.8 ± 1.7
ΣC≥,ω-3	3.4 ± 1.0	4.2 ± 0.6	4.9 ± 0.8	5.8 ± 1.1	8.0 ± 0.7†	8.0 ± 1.2	5.2 ± 3.0	4.4 ± 0.4	7.7 ± 0.7	8.3 ± 2.0	4.4 ± 0.6	6.9 ± 0.6
X Chain												
Lth	18.0 ± 0.0	17.8 ± 0.1	18.2 ± 0.1	18.3 ± 0.1	18.0 ± 0.1	18.0 ± 0.1	18.1 ± 0.1	18.1 ± 0.4	18.0 ± 0.0	18.0 ± 0.2	18.1 ± 0.2	18.4 ± 0.1
UI	102 ± 10	118 ± 12	142 ± 8	161 ± 9	141 ± 5	141 ± 5	154 ± 13	150 ± 13	144 ± 4	153 ± 12	140 ± 14	167 ± 7

Data are given as means + SE by age and sex for Inuit and Vancouver residents (Table 2) for major fatty acids carbon chain length ≥ 14.

*, † indicate values for Inuit males or females significantly different $p \leq 0.05, 0.01$, respectively, from value for Vancouver males or females, respectively.

‡ indicates value for Inuit female significantly different, $p < 0.01$ from Inuit male of similar age.

UI, unsaturation index.

TABLE 5

Serum Cholesterol Levels

Age yrs	Inuit Male (mg/dl	Inuit Female mg/dl	Vancouver Male mg/dl	Vancouver Female mg/dl
0-5	118 ± 8			
6-10	147 ± 11			
11-15	161 ± 12	148 ± 20		
16-20	177 ± 17	174 ± 15		
21-50	180 ± 42	183 ± 25	197 ± 22	185 ± 15
>50	184 ± 43	201 ± 28		

Data are given as means ± SE by age and sex for Inuit and Vancouver residents (Table 2). No statistically significant ($p \leq 0.05$) differences were found between male and female Inuit or Inuit and Vancouver residents in a similar age group.

The plasma PL of Greenland Eskimo has been reported to contain greatly decreased levels of 18:2n-6 and increased 20:5n-3 and 22:6n-3 compared with Eskimo living in Denmark (5). Similar analyses of Japanese who consumed diets high in fish found levels of 20:5n-3 ca. 2-fold higher, and 22:6n-3 ca. 2-fold lower, than in Greenland Eskimo (28). These differences between the Eskimo and Japanese may reflect, at least in part, their dietary n-3 fatty acid sources. Important differences have been shown in plasma PL n-6 and n-3 series fatty acids between Eskimo living in Denmark and Danes (5), Japanese and North Americans (28) and West Coast Canadian Indians and Europeans and North Americans (29), and may suggest that important racial variations exist in essential fatty acid metabolism (29,30). In the Broughton Island Inuit, red cell 20:4n-6 was reduced in PE; however, the level of this fatty acid in PC, and of 18:2n-6 in male and female PE and male PC was similar to the Vancouver reference group. Thus, diet rather than racial differences in fatty acid metabolism, appears to be the major determinant of red cell composition in the Canadian Inuit.

The serum cholesterol analyses found no difference in the Inuit due to sex, and no difference from Vancouver residents of similar age (Table 5). Values for the Inuit serum cholesterol concentrations are lower than in previous studies of Alaskan Eskimo adults (31) and Eskimo adults and children (32). The latter study also noted no difference between Eskimo males and females.

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REFERENCES

1. Bang, H.O., and Dyerberg, J. (1972) *Acta Med. Scand. 192,* 85-94.
2. Bang, H.O., Dyerberg, J., and Hjorne, N. (1976) *Acta Med. Scand. 200,* 69-73.
3. Bang, H.O., Dyerberg, J., and Nielsen, A.B. (1971) *Lancet 1,* 1143-1146.
4. Bang, H.O., Dyerberg, J., and Sinclair, H.M. (1980) *Am. J. Clin. Nutr. 33,* 2657-2661.
5. Dyerberg, J., Bang, H.O., and Hjorne, N. (1975) *Am. J. Clin. Nutr. 28,* 958-966.
6. Knapp, H.R., Reilly, I.A.G., Alessandrini, P., and Fitzgerald, G.A. (1986) *New Engl. J. Med. 314,* 937-942.
7. von Schacky, C., Fischer, S., and Weber, P.C. (1985) *J. Clin. Invest. 76,* 1626-1631.
8. von Schacky, C., and Weber, P.C. (1985) *J. Clin. Invest. 76,* 2446-2450.
9. Cartwright, I.J., Pockley, A.G., Galloway, J.H., Greaves, M. and Preston, F.E. (1985) *Atherosclerosis 55,* 267-281.
10. Popp-Snijders, C., Schouten, J.A., van der Veen, J., and van der Veen, E.A. (1986) *Scand. J. Clin. Lab. Invest. 46,* 253-258.
11. Popp-Snijders, C., Schouten, J.A., van Blitterswijk, W.J., and van der Veen, E.A. (1986) *Biochim. Biophys. Acta 854,* 31-37.
12. Kromhout, D., Bosschieter, E.B., and Coulander, C. (1985) *New Engl. J. Med. 312,* 1205-1209.
13. Shekelle, R.B., Missel, L.V., Oglesby, P., Shryolk, A.M., and Stamler, J. (1985) *New Engl. J. Med. 313,* 820.
14. Curb, J.D., and Reed, D.M. (1985) *New Engl. J. Med. 313,* 821-822.
15. Norell, S.E., Ahlbom, A., and Feychting, M. (1986) *Br. Med. J. 293,* 426.
16. Vollset, S.E., Heuch, I., and Bjelke, E. (1985) *New Engl. J. Med. 313,* 820-821.
17. Innis, S.M., and Kuhnlein, H.V. (1987) *Acta Med. Scand. 222,* 105-109.
18. Exler, J., and Weihrauch, J.L. (1976) *J. Am. Diet. Assoc. 69,* 243-248.
19. Puustinent, T., Punnonen, K., and Uotila, P. (1985) *Acta Med. Scand. 218,* 59-62.
20. Ackman, R.G. (1988) *Atherosclerosis 70,* 171-173.
21. Dougherty, R.M., Galli, C., Ferro-Luzzi, A., and Iacono, J.M. (1987) *Am. J. Clin. Nutr. 45,* 443-455.
22. Farquhar, J.W., and Ahrens, E.H. (1963) *J. Clin. Invest. 42,* 675-685.
23. Reed, C.F., Swisher, S.N., Marinetti, G.V., and Eden, E.G. (1960) *J. Lab. Clin. Med. 56,* 281-289.
24. Innis, S.M., and Clandinin, M.T. (1981) *Biochem. J. 193,* 155-167.
25. Skipski, V.P., Peterson, R.F., and Barclay, M. (1964) *Biochem. J. 90,* 374-377.
26. Cohen, P., and Derksen, A. (1969) *Br. J. Haemat. 17,* 359-371.
27. Manku, M.S., Horrobin, D.F., Huang, Y.S., and Morse, N. (1983) *Lipids 18,* 906-908.
28. Yamori, Y., Nara, Y., Iritani, N., Workman, R.J., and Inagami, T. (1985) *J. Nutr. Sci. Vitaminol. 31,* 417-422.
29. Bates, C., van Dam, C., Horrobin, D.F., Morse, N., Huang, Y-S., and Manku, M.S. (1985) *Prostaglan. Leukotr. Med. 17,* 77-84.
30. Horrobin, D.F. (1987) *Med. Hypoth. 22,* 421-428.
31. Wilder, C.G., and Levine, V.E. (1950) *Exp. Med. Surg. 8,* 422-425.
32. Feldman, S.A., Ho, K-J., Lewis, L.A., Mikkelson, B., and Taylor, B. (1972) *Arch. Path. 94,* 42-58.

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Formation of Fluorescent Substances From Degradation Products of Methyl Linoleate Hydroperoxides With Amino Compound

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The degradation products formed from methyl linoleate hydroperoxides by reaction with heme were fractionated by Sephadex LH-20 column chromatography and by reverse-phase high performance liquid chromatography, and the ability of each compound to form fluorescent substances through reaction with amino compound was compared. Maximum formation of fluorescent substances was obtained from monomeric degradation products with amino compound, but low molecular weight aldehydes such as hexanal, 2-hexenal and 2,4-decadienal, formed only a small amount of fluorescent substances. However, the major monomeric degradation products described previously, the hydroxy-, keto- and epoxy-derivatives, do not significantly contribute to the formation of fluorescent substances through reaction with amino compound. It was suggested that formation of fluorescent substances from lipid peroxides with amino compound may originate from a precursor present in monomeric degradation products formed from hydroperoxide of methyl linoleate during lipid peroxidation, and that low molecular weight aliphatic aldehydes are not involved in fluorescent substance formation. Moreover, the majority of TBA-reactive substances in secondary oxidation products prepared from autoxidized methyl linoleate are also unrelated to the formation of fluorescent substances through reaction with amino compound.
Lipids 23, 1069–1072 (1988).

Lipid peroxides of polyunsaturated fatty acids (PUFA) play an important role in the formation of fluorescent lipofuscin pigments, which are associated with aging in animal tissues. Reviews on the formation and characterization of fluorescent lipofuscin pigments in vivo or in vitro have been reported recently by Kikugawa (1) and Tsuchida et al. (2).

The degradation products of methyl linoleate (ML)-hydroperoxide (HPO) produce fluorescent substances (FS) with spectra similar to lipofuscin pigments, exhibiting excitation and emission maxima at 320–360 nm and 410–430 nm upon reaction with glycine or DNA (3,4). We have also reported that both 9- and 13-positional ML-HPO produce FS having the same fluorescence spectra upon reaction with 1-aminopentane (1-AP) as a model amino compound in the presence of heme (5). The degradation of PUFA-HPO by catalysis of heme compounds, metals or ascorbic acid leads to the production of very complicated compounds, including monomeric hydroxy-, keto- or epoxy-derivatives (6–9) and low molecular weight aldehydes such as alkanals, 2-alkenals, 2,4-alkadienals or malondialdehyde (10,11). It is well known that these low molecular weight aldehydes form FS by reaction with various amino compounds (12–16). However, Frankel et al. have recently reported that methyl linolenate HPO is readily decomposed thermally at 150°C and catalytically with ferric chloride-ascorbic acid at room temperature, producing dimeric or monomeric compounds and volatile compounds including low molecular weight monoaldehydes (17). Also, monomeric compounds were shown to exhibit marked formation of FS through reaction with DNA (18). We recently reported that secondary oxidation products (SP) prepared from autoxidized ML, which is a complex mixture of various oxidized products converted from ML-HPO, also produce similar FS directly through reaction with 1-AP (19).

These results suggest that oxidative degradation of PUFA-HPO is necessary for formation of FS through reaction with various amino compounds. However, the direct precursor available for formation of FS has not yet been determined from among the many kinds of degradation products of PUFA-HPO. In this study, therefore, the degradation products formed from ML-HPO by reaction with heme were fractionated by Sephadex LH-20 column chromatography and by reverse-phase high performance liquid chroamtography (HPLC). The results obtained regarding the characterization of the precursor available for formation of FS through reaction with amino compound are discussed.

MATERIALS AND METHODS

Materials. Linoleic acid, dilinolein, hemin and soybean lipoxygenase were purchased from Sigma Chemical Co. (St. Louis, MO). 1-AP, ML and hexanal were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). 2-Hexenal and 2,4-decadienal were obtained from Aldrich Chemical Co. (St. Louis, MO). Heme methyl ester (heme) was prepared by the method of Ortiz de Montellano et al. (20).

Preparation of HPO and SP from ML. ML (3.0 g) was autoxidized by stirring at 30°C for 5 or 14 days by methods described previously (19). ML-HPO reached a maximum at 4–5 days and was prepared by silica gel column chromatography (2 × 40 cm, Wako gel C-200, Wako Pure Chemical Industries Ltd., Osaka, Japan) with hexane/ethyl ether (7:3) according to the method of Terao and Matsushita (21). Crude SP, including the precursor available for formation of FS, was also prepared from ML autoxidized for 14 days by the same method as that described above (19). [1-^{14}C]ML-HPO was prepared from [1-^{14}C]linoleic acid (New England Nuclear, Boston, MA) by autoxidation. Each HPO was estimated using a molar absorbance value of 24,500 at 233 nm (22).

Fractionation of degradation products of ML-HPO or crude SP. ML-HPO (100 μmol) was incubated with or without heme (1 μmol) in 100 ml of methanol at 37°C for 30 min. The degradation products were subjected to Sephadex LH-20 column chromatography (1 × 40 cm,

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Abbreviations: HPO, hydroperoxide; ML, methyl linoleate; 1-AP, 1-aminopentane; TBA, thiobarbituric acid; HPLC, high performance liquid chromatography; FS, fluorescent substance; GC, gas chromatography; MS, mass spectrm or spectrometry; PUFA, polyunsaturated fatty acids; SP, secondary oxidation products.

Pharmacia Fine Chemicals, Uppsala, Sweden) according to the method of Kanazawa et al. (23) and were eluted with ethanol. Two-ml fractions were collected and monitored at 233 nm and 278 nm. Crude SP (50 mg) was also subjected to the same column chromatography and the effluents were monitored by measuring the thiobarbituric acid (TBA) values. The degradation products from labeled or nonlabeled ML-HPO were also subjected to reverse-phase HPLC on a μ-Bondapak FAA (3.9 × 300 mm, Waters Associates, Milford, MA) using acetonitrile/methanol/water (20:45:55) for the first 30 min followed by methanol as the mobile phases at a flow rate of 1.0 ml/min. The effluents were monitored by absorbance at 254 nm and by ^{14}C radioactivity. Each fraction was collected and the components extracted with chloroform/methanol (2:1) were used for formation of FS and structural analysis. The electron ionization mass spectrum (MS) was measured with a JEOL-D300 mass spectrophotometer using a 30-eV ionization voltage. Gas liquid chromatography was carried out using a glass column packed with 5% silicon SE-52 on celite 545 SK DMCS (Gasukuro Kogyo Inc., Tokyo) at a column temperature of 250°C. The UV absorption spectra were measured with a Hitachi 200-10 spectrophotometer.

Formation of fluorescent substances. ML-HPO (1 μmol) preincubated with or without heme (10 nmol) was incubated with 1-AP (2 μmol) in 3 ml of methanol at 37°C for 20 hr. Hexanal, 2-hexenal or 2,4-decadienal was also incubated with 1-AP in 3 ml of methanol at 37°C for 20 hr. Formation of FS from ML-HPO or these aldehydes with 1-AP was observed to be marked under these conditions. The degradation products in each fraction from HPLC were also incubated with 1-AP under the above conditions. Fluorescence spectra were measured with a Hitachi MPF-3 fluorescence spectrophotometer and fluorescence intensities were expressed as a percentage of that of a quinine sulfate standard (0.1 μg/ml of 0.1 N H_2SO_4). HPLC was carried out on a μ-Bondapak FAA (3.9 × 300 mm, Waters) preceded by a packed guard column (4 × 50 mm, Unisil C_{18}, 10 μm, Gasukuro Kogyo Inc.) with tetrahydrofuran/acetonitrile/water (15:45:50) at a flow rate of 1.0 ml/min. An aliquot of each FS was subjected to the same HPLC conditions and was monitored at excitation and emission maxima of 350 nm and 420 nm with an Hitachi 650-10LC fluorescence spectrophotometer.

RESULTS AND DISCUSSION

ML-HPO produced FS by reaction with 1-AP in the presence of heme, and the same two major fluorescence peaks (FS-I and FS-II) were eluted on reverse-phase HPLC as those formed from SP with 1-AP (5). The degradation products formed from ML-HPO by reaction with heme were fractionated by Sephadex LH-20 column chromatography and then the ability to form FS through reaction with 1-AP in each fraction was studied. The absorption peaks monitored at 233 nm and 278 nm, respectively, were detected in nearly the same fraction, 12–15, but maximum formation of fluorescent substances was obtained from Fr. 9–13. Based on the report by Kanazawa et al. (23), Fr. 9–13 contains mainly polymer- and endoperoxide-rich components, and Fr. 14–18 contains low molecular weight components. Marker compounds, 2-hexenal, 2,4-decadienal, ML-HPO and dilinolein (molecular weight: 617) were eluted at the positions shown by arrows in Figure 1. Oarada et al. (24) separated autoxidized ML by gel chromatography on Bio Beads S-X3 into polymeric, monomeric and low molecular weight compounds. In our study, the elution profiles of the degradation products produced from ML-HPO showed that the precursor available for formation of FS is contained in Fr. 9–13 (Fig. 1) and that it is one of the monomeric degradation products rather than a low molecular weight compound.

When the degradation products produced from [1-^{14}C]ML-HPO by reaction with heme were subjected to reverse-phase HPLC, seven radioactive peaks were detected (Fig. 2). From the UV spectral analysis and gas chromatography–mass spectrometry (GC–MS) fragmentations of the major degradation products described in a previous report (5), the structures produced from 13-HPO of ML were found to be methyl 9-hydroxy-12,13-epoxylinoleate and methyl 11-hydroxy-12,13-epoxylinoleate (17.2%, Fr. II) having no UV absorption, methyl 9-keto-12,13-epoxylinoleate (13.4%, Fr. III), methyl 13-hydroxylinoleate (3.3%, Fr. IV), 13-HPO (9.7%, Fr. V) and methyl 13-ketolinoleate (4.6%; Fr. VI). Similarly, each fraction also contained the analogous derivatives produced from 9-HPO of ML. The major degradation products have been described previously by Hamberg (7) and Dix and Marnett (8). In this experiment, 2-hexenal and 2,4-decadienal as marker compounds were detected at fraction numbers 5 and 10, respectively. When the fractions (Fr. I–VII) detected by HPLC were compared for their ability to form FS by reaction with 1-AP, the most marked formation of FS-II was shown in Fr. VII. This

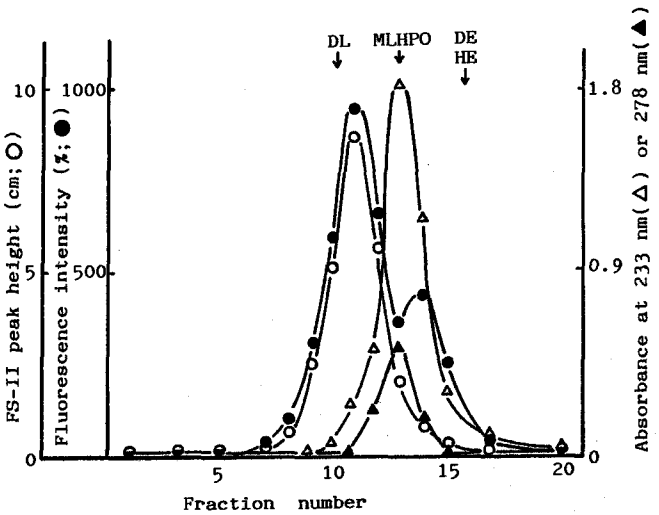

FIG. 1. Sephadex LH-20 column chromatography of the degradation products produced from ML-HPO by reaction with heme. The degradation products of ML-HPO were obtained from reaction of ML-HPO (100 μmol) with heme (1 μmol) at 37°C for 30 min. Sephadex LH-20 column chromatography was carried out according to the experimental method. The effluents were monitored at 233 nm (△) and 278 nm (▲). An aliquot of each fraction was incubated with 1-AP at 37°C for 20 hr and then total fluorescence intensity (●) and FS-II peak height (O) were measured as described in Materials and Methods. 2-Hexenal (HE), 2,4-decadienal (DE), ML-HPO and dilinolein (DL) as marker compounds were eluted at the positions shown by arrows.

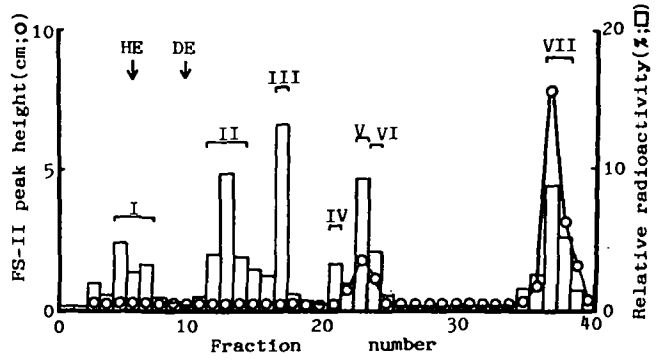

FIG. 2. HPLC of the degradation products produced from ML-HPO by reaction with heme. The degradation products from [1-^{14}C]ML-HPO by reaction with heme were subjected to reverse-phase HPLC. The effluents were monitored by radioactivity (□) and each fraction was incubated with 1-AP for formation of fluorescent substances. FS-II peak height (○) was measured by HPLC analysis as described in Materials and Methods. The structure of each component was identified by a combination of UV analysis and GC-MS fragmentation by the methods described in the previous report (5).

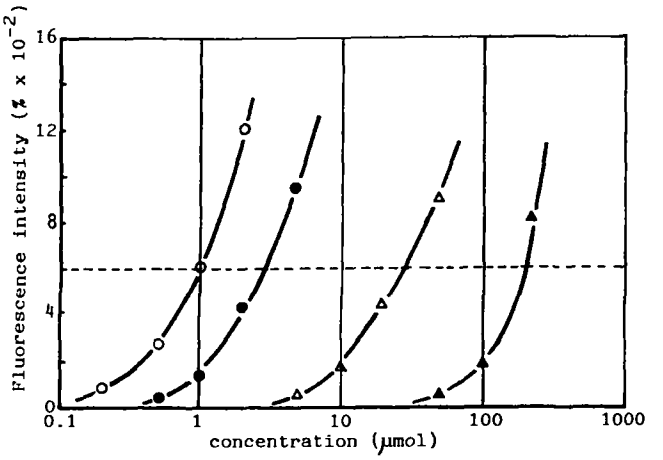

FIG. 3. Comparison of the ability for formation of fluorescent substances by ML-HPO and aldehydes through reaction with 1-AP. ML-HPO (○) was preincubated with heme and then incubated with 1-AP for formation of fluorescent substances in 3 ml of methanol at 37°C for 20 hr. 2,4-Decadienal (●), 2-hexenal (△) or hexanal (▲) also was incubated with 1-AP under the same conditions. Total fluorescence intensity and fluorescence spectrum of each reaction mixture were measured as described in Materials and Methods.

result suggests that the major monomeric degradation products, hydroxy-, keto- and epoxy-derivatives in Fr. II-VI, are not significantly related to the formation of FS through reaction with amino compound. Moreover, monofunctional aldehydes such as 2-hexenal and 2,4-decadienal also do not form FS. The direct precursor available for most of the formation of FS was included in Fr. VII. This fraction accounted for about 13.3% of the total degradation products produced from radioactive ML-HPO. We were unable to identify the components in Fr. I (10.8%) and VII (13.3%) in this study. Fukuzawa et al. (25) reported the formation of FS from 12-keto-oleic acid with amino acids in 0.1 M phosphate buffer (pH 7.4). In our study, methyl 13-keto-linoleate (Fr. VI) also formed a slight FS, but this compound was not the direct precursor for formation of FS. 11-Methoxy-12,13-epoxylinoleate was formed from ML-HPO by acid catalysis (26), but this compound also showed slight formation of FS (data not shown).

We next compared the ability to form FS through reaction with 1-AP between the degradation products of ML-HPO and aliphatic monofunctional aldehydes (Fig. 3). One μmol of ML-HPO showed marked fluorescence intensity (600%) with 1-AP in the presence of heme, but hexanal and 2-hexenal produced only very small amounts of FS at the same concentration. 2,4-Decadienal was more active than 2-hexenal and hexanal, but still less active than ML-HPO. 2,4-Decadienal, 2-hexenal and hexanal subsequently required 2.7-, 28.8- and 175-fold larger amounts, respectively, to obtain the same fluorescence intensity (600%) as that from ML-HPO (Table 1). Although it has been reported that monofunctional aliphatic aldehydes produce FS through reaction with various amino compounds at a high molar concentration (15,16), the amounts of these aldehydes derived from PUFA-HPO account for only a few percent of the degradation products formed from their HPO (17). These results indicate that the aliphatic aldehydes described in this study and the structurally related monomeric degradation products are not direct precursors available for formation of FS through reaction with amino compounds.

TABLE 1

Relationship Between Fluorescence Intensity Formed From ML-HPO or Aldehydes With 1-AP and Their Concentration

Compound	μmol	Fluorescence maxima (nm) Excitation	Emission	intensity (%)
ML-HPO	1.0	348	420	600.0
2,4-Decadienal	1.0	340	416	140.0
	2.7	346	422	600.0
2-Hexenal	1.0	—	—	ND
	28.8	344	408	600.0
Hexanal	1.0	—	—	ND
	175.0	366	442	600.0

ML-HPO was preincubated with heme and then incubated with 1-AP at 37°C for 20 hr. Each aldehyde also was incubated with 1-AP at 37°C for 20 hr.

ND, fluorescence not detected.

When SP prepared from autoxidized ML was subjected to the same Sephadex LH-20 column chromatography, a high TBA value was detected in Fr. 10–15. Therefore, each of the fractions was incubated with 1-AP at 37°C for 20 hr to form FS, and the total fluorescence intensity was observed in Fr. 8–14, whereas a major FS (FS-II) was detected in Fr. 8–10 (Fig. 4). This result suggests that the greater proportion of the TBA-reactive substances (Fr. 10–15) are not related to formation of major FS from SP with amino compound. We have also demonstrated that large amounts of TBA-reactive substances prepared from crude SP using a Sep-pak C_{18} cartridge were not related to the formation of FS (19). Similarly, when SP was subjected to the same reverse-phase HPLC, marked formation of FS-II was observed in Fr. VII eluted with methanol (data not shown). These results indicate that SP also contains a precursor available for the formation

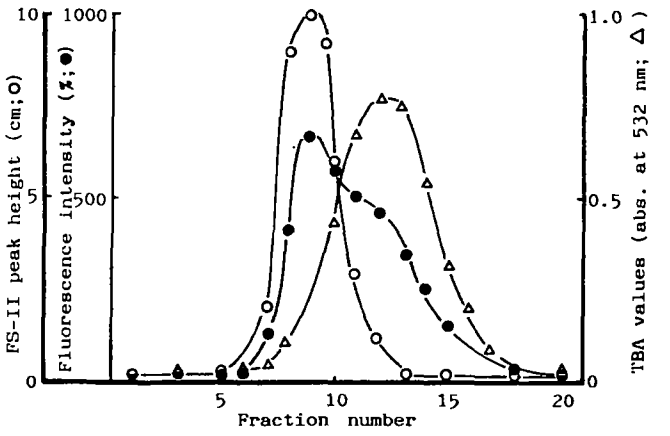

FIG. 4. Sephadex LH-20 column chromatography of SP. SP (50 mg) prepared from autoxidized ML was subjected to Sephadex LH-20 column chromatography. The effluents were monitored by measurement of TBA values (△) and an aliquot of each fraction was incubated with 1-AP for formation of fluorescent substances in 3 ml of methanol at 37°C for 20 hr. Total fluorescence intensity (●) and FS-II peak height (○) were measured using the same methods as described in Figure 1. Marker compounds were eluted at the same positions shown in Figure 1.

of FS through reaction with amino compound, similar to that from ML-HPO described above.

Each FS (FS-II) formed from SP or ML-HPO with 1-AP in methanol solution was shown to have an empirical formula $C_{24}H_{41}NO_4$, with a molecular weight of 407 (data not shown). The other characteristic fragmentation ions ($M^+ - 31$, $M^+ - 57$, $M^+ - 71$, $M^+ - 143$ and $M^+ - 157$) indicate the presence of a pentyl group and methyl octanoate in FS-II. This suggests that the direct precursor available for formation of FS may be a monomeric 19-carbon compound having a methyl ester group produced from ML without cleavage into low molecular weight compounds. We also demonstrated that HPO of 1-palmitoyl-2-linoleoyl-phosphatidylcholine and trilinolein produced FS through reaction with 1-AP after degradation by heme and that the FS (FS-II) was released from their fluorescent lipids after transmethylation (27). This result also suggests that the direct precursor available for formation of FS is a monomeric degradation product attached to a phosphatidylcholine glycerol backbone without breakdown into low molecular weight compounds.

PUFA are converted into very complicated oxidative degradation products, including dimeric or monomeric compounds and low molecular weight aldehydes during the peroxidation process (10,11). Malondialdehyde and aliphatic aldehydes are known to produce FS by reaction with amino compound (12–16). Moreover, Benedetti et al. (28) and Yoshioka and Kaneda (29) have reported that 4-hydroxy, 4,5-dihydroxy or 4-hydroperoxy alkenals show marked toxic effects on the enzyme activities of animal tissues. Esterbauer et al. have suggested the possible involvement of 4-hydroxynonenal in the formatio of fluorescent chromolipids (30). These reports suggest that various aldehydes may be related to the formation of FS through reaction with amino compound during the process of peroxidation. However, our present results strongly indicate that the direct precursor available for formation of FS from lipid peroxides with amino compound is a monomeric degradation product formed from PUFA-HPO rather than low molecular weight aliphatic aldehydes. Moreover, the major monomeric degradation products produced from 9- and 13-HPO of ML described previously (6–9) do not directly produce FS through reaction with amino compound. The identity of this direct precursor is now under investigation.

REFERENCES

1. Kikugawa, K. (1986) *Adv. Free Rad. Biol. Med. 2,* 389–417.
2. Tsuchida, M., Miura, T., and Aibara, K. (1987) *Chem. Phys. Lipids 44,* 297–325.
3. Shimasaki, H., and Privett, O.S. (1977) *J. Am. Oil Chem. Soc. 54,* 119–123.
4. Fujimoto, K., Neff, W.E., and Frankel, E.N. (1984) *Biochim. Biophys. Acta 795,* 100–107.
5. Iio, T., Yoden, K., and Tabata, T. (1987) *Chem. Pharm. Bull. 35,* 5015–5019.
6. Gardner, H.W., and Kleiman, R. (1981) *Biochim. Biophys. Acta 665,* 113–125.
7. Hamberg, M. (1983) *Biochim. Biophys. Acta 752,* 191–197.
8. Dix, T.A., and Marnett, L.J. (1985) *J. Biol. Chem. 260,* 5351–5357.
9. Gardner, H.W. (1987) in *Autoxidation of Unsaturated Lipids* (Chan, H.W.-S., ed.) 51–93, Academic Press, London, England.
10. Frankel, E.N. (1984) *J. Am. Oil Chem. Soc. 16,* 1908–1917.
11. Grosch, W. (1987) in *Autoxidation of Unsaturated Lipids* (Chan, H.W.-S., ed.) pp. 95–139, Academic Press, London, England.
12. Chio, K.S., and Tappel, A.L. (1969) *Biochemistry 8,* 2821–2827.
13. Kikugawa, K., Maruyama, T., Machida, Y., and Kurechi, T. (1981) *Chem. Pharm. Bull. 29,* 1423–1432.
14. Beppu, M., Murakami, K., and Kikugawa, K. (1986) *Chem. Pharm. Bull. 34,* 781–788.
15. Hasegawa, K., Fujimoto, K., Kaneda, T., and Frankel, E.N. (1987) *Lipid Peroxide Res. J. 11,* 82.
16. Kikugawa, K., and Sawamura, A. (1987) *J. Am. Oil Chem. Soc. 64,* 1156–1162.
17. Frankel, E.N., Neff, W.E., Selke, E., and Brooks, D.D. (1987) *Lipids 22,* 322–327.
18. Frankel, E.N., Neff, W.E., Brooks, D.D., and Fujimoto, K. (1987) *Biochim. Biophys. Acta 919,* 239–244.
19. Iio, T., and Yoden, K. (1987) *Life Sci. 40,* 2297–2302.
20. Ortiz de Montellano, R.P., and Mico, B.A. (1980) *Mol. Pharmacol. 18,* 128–135.
21. Terao, J., and Matsushita, S. (1977) *J. Am. Oil Chem. Soc. 34,* 234–238.
22. Johnston, A.E., Zilch, K.T., Selke, E., and Dutton, H.J. (1961) *J. Am. Oil Chem. Soc. 38,* 367–371.
23. Kanazawa, K., Danno, G., and Natake, M. (1983) *Agr. Biol. Chem. 47,* 2035–2043.
24. Oarada, M., Miyashita, T., and Kaneda, T. (1986) *Lipids 21,* 150–154.
25. Fukuzawa, K., Kishimoto, K., Tokumura, A., Tsukatani, H., and Shibuya, M. (1985) *Lipids 20,* 854–861.
26. Gardner, H.W., Weisleder, D., and Nelson, E.C. (1984) *J. Org. Chem. 49,* 508–515.
27. Iio, T., and Yoden, K. (1988) *Lipids 23,* 65–67.
28. Benedetti, A., Comporti, M., and Esterbauer, H. (1980) *Biochim. Biophys. Acta 620,* 281–296.
29. Yoshioka, M., and Kaneda, T. (1972) *Yukagaku J. 21,* 316–321.
30. Esterbauer, H., Koller, E., Slee, R.G., and Koster, F. (1986) *Biochem. J. 239,* 405–409.

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Predicting Coordinated Lipid Biosynthesis: Application to the Surfactant-accommodated Epidermis

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Factorialized correlation analysis is proposed as a method for predicting the coordination of multiple enzyme pathways. The approach can be used potentially to find new relationships and to predict relationships that have been established in other tissues. However, careful tracer studies are needed to verify the cause-and-effect relationships between precursor and products. In this study, guinea pigs that were chronically treated with an anionic, a nonionic and a cationic surfactant passed through an irritation stage to a clinical state that appeared normal. The method was used to examine binary coordination of lipid biosynthesis in the epidermis by using a factorialized table of regression coefficients. Coordinated lipid relationships that have been reported in other tissues were predicted between sphingomyelin and cholesterol, as well as between phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine. A new inverse relationship was found between triglycerides and both sphingomyelin and cholesterol, using this method. These data are discussed with respect to a membrane fluidization model for the accommodated state.
Lipids 23, 1073–1078 (1988).

Mechanistic interpretations of biosynthetic coordination are often confounded by an abundance of analytical information. We propose that the degree of coordination between two different components can be estimated from correlation analysis. The only requirement is that reproducible conditions must be found for changing the components in the tissue so that at least three different levels are found for each component.

In this study, the lipid levels were changed in the epidermis of guinea pigs by treating the animals with three different surfactants until an accommodation response was found. An accommodation response is an increased local resistance to a primary irritant after a period of irritation. Repeated contact with the irritant is necessary to maintain this condition (1,2). The lipids in the accommodated epidermis were altered so that the surfactant-and-water-control treatments provided four different levels of each lipid for comparison. Correlation analysis was carried out between pairs of lipids with respect to the different treatments.

MATERIALS AND METHODS

Surfactants. Three different classes of surfactants were used in this study: anionic—sodium lauryl sulfate (Henkel Inc., Fort Lee, NJ), nonionic—laureth-1 (Lipo Chemicals Inc., Paterson, NJ) and cationic—laurtrimonium chloride (Henkel Inc.). The same alkyl chain was chosen so that any adaptive differences that might occur would be due to the surfactant head group.

Animals. Male Hartley guinea pigs, 250–300 g, were accommodated by treating them daily with an aqueous surfactant solution according to the method of Opdyke and Burnett (3). The adaptive concentration used for sodium lauryl sulfate (SLS) and laurtrimonium chloride (LTC) was 0.15%. A much higher concentration of 1.2% was necessary for laureth-1 (L-1). The water bath was maintained at 40°C as recommended. At this temperature, the animals are calm and tolerate the treatment well. Each animal was treated for 2 hr a day for 4–6 wk. After a treatment, the animals were thoroughly rinsed with warm tap water and toweled dry. At the time the animals were killed, they weighed 500–800 g.

Epidermal cell suspension. A modified version of the method of Stanley et al. (4) was used to obtain an epidermal cell suspension from abdominal and back skin. After 1-cm-wide strips of epidermis were separated from the dermis by trypsinization, the epidermis was placed in 10 ml of 0.9% saline with 0.005% soybean-trypsin inhibitor and vortexed for 5 min on a Vortex Geni. These cells were then filtered through a 500-μm nylon mesh and centrifuged at 200 × *g* for 20 min in a 35 ml round-bottom centrifuge tube fitted with a teflon-lined cap. The cell pellet was washed, centrifuged twice more and finally suspended in 1.6 ml of saline.

Lipid extraction. Extraction was carried out by the method of Bligh and Dyer (5) using their recommended volume ratios of chloroform, methanol and aqueous solution. The final chloroform layer was obtained from a 25-ml separatory funnel after the extract had been centrifuged at 200 *g* for 15 min to reduce the emulsified layer.

Lipid separation and quantitation. The chloroform extract was evaporated to dryness under a gentle stream of nitrogen and reconstituted to 100 μl. A 10-μl TLC syringe was then used to spot 1-μl aliquots onto Silica Gel G plates (250 μm × 20 cm × 20 cm) that had been previously predeveloped in solvent A (chloroform/methanol/glacial acetic acid/water, 25:15:4:1.9, v/v/v/v) and solvent B (hexane/diethyl ether/glacial acetic acid, 83:16:1, v/v/v) to reduce charring background. Plates were developed to 12 cm in solvent system A. After they were dried, they were developed to the top of the plate in solvent system B. The first solvent system separated phospholipids. The second separated neutral lipids. Commercially available lipids were used to construct standard curves and to provide an R_f comparison for native lipids. Chloroform stock solutions of lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, oleic acid, stearic acid, triolein, tripalmitin, tristearin, cholesteryl oleate, cholesteryl palmitate, cholesteryl stearate and cholesterol were used for these purposes.

Quantitation was carried out using the liquid scintillation counter to assay charred lipids (6). After the plates were sprayed with a 10% (w/v) cupric sulfate 8% (w/v)

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Abbreviations: L-1, laureth-1; LTC, laurtrimonium chloride; SLS, sodium lauryl sulfate; AEC, automatic external standard channels ratio; TLC, thin layer chromatograph(ic,y).

orthophosphoric acid solution, they were placed in a forced-draft hot air oven for 30 min at 180°C. Charred lipid spots were scraped into 7-ml scintillation vials and vortexed in 800 μl of water to disperse the particles. Five ml of ScintiVerse II liquid scintillation cocktail (Fisher Scientific, Fair Lawn, NJ) was added, and the vial was then vortexed to achieve a uniform dispersion in the formed hydrogel. A brief centrifugation period was used to remove any bubbles. Samples were counted on a Packard Tri-Carb Model 3309 liquid scintillation spectrometer using the automatic external standard channels ratio number for quantitation (7–11).

Correlation analysis. If normal precursor-product relationships between different lipids occur independent of the stress placed on the epidermis, a linear regression analysis between different lipid pairs for the four different treatments (water, anionic, nonionic and cationic) should show a high degree of correlation. On the other hand, a poor correlation would indicate a lack of synchrony. For example, a linear regression was carried out between sphingomyelin and phosphatidylserine with respect to the four different treatments. The correlation coefficient, r, for this pair was +0.24, a poor positive correlation. This indicates that mutual changes between these two lipids did not occur in a highly synchronous manner. For perfect synchrony, a correlation coefficient of 1.0 is needed. Thus, an increase (or decrease) in sphingomyelin, for every treatment, is matched by an exact proportional increase (or decrease) in phosphatidylserine. It is this degree of linear proportionality between these changes that governs the strength of the correlation coefficient.

Negative correlations, on the other hand, occur when the treatments cause paired lipid changes to be inversely proportional to one another. For example, the correlation between sphingomyelin and triglycerides gave a correlation coefficient of −0.98. This means that increases (or decreases) in sphingomyelin occurred in synchrony with decreases (or increases) in triglycerides in a nearly exact inversely proportional manner.

RESULTS

Accommodative irritation. Initial studies showed that the degree of irritation caused by the nonionic surfactant L-1 was much less than either SLS or LTC, the anionic and cationic surfactants, respectively. By using a 1.2% solution of L-1, it was found that the degree and duration of irritation was visually equivalent to that caused by a 0.15% solution of SLS or LTC. During the irritation phase of the treatment, the skin turned red and started to crack after 2–3 days. This phase was followed by 2–3 days of dry and flaky skin. Finally, after 2 or 3 wk, the skin and fur appeared normal for all three surfactants.

Histologically, however, there were great differences. These are apparent in Figure 1. In comparison with a water-treated control, the SLS-accommodated skin appeared the most normal. A slight acanthosis and minor granular layer activity were apparent. In contrast, the nonionic- and cationic-accommodated skin was hyperplastic, the prickle-cell layer being 2–4 times as thick as the SLS-adapted skin. However, the hyperplasia, in all cases, showed no neoplastic changes because it was general and diffuse and not focal. An absence of mutagenesis of the tissue or cell-type transformation was apparent from a lack of irregular nuclei or large cells and from the presence of a regular orderly basal layer. A few mitotic figures and lymphocytes were present in the basal layer of the epidermis and in the dermis, respectively, of the L-1- and LTC-treated animals, indicating some residual inflammation. In addition, the dermis of these animals had sebaceous glands that were enlarged and engorged with cells.

Lipid quantitation. The thin layer chromatographic (TLC) system separated 4 polar lipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin), as well as 2 neutral lipid groups (cholesteryl esters and triglycerides). The extract also resolved a lipid that was more polar than sphingomyelin. This lipid was presumed to be a lysophospholipid. The

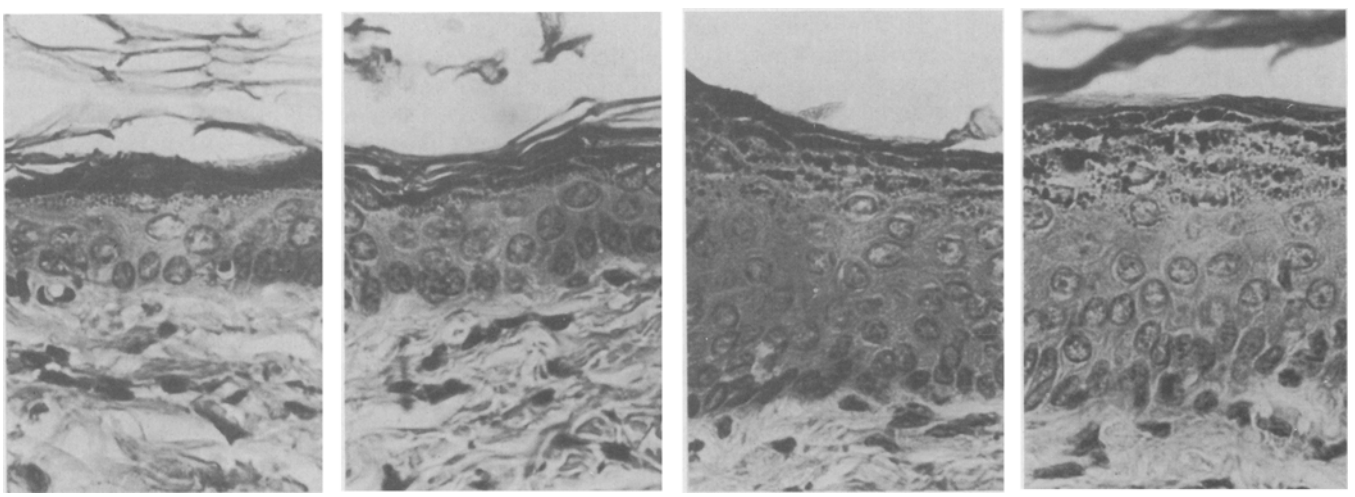

FIG. 1. Adapted epidermises of guinea pigs treated at 40°C for 4–6 weeks. A = water treatment, B = 0.15% sodium lauryl sulfate, C = laureth-1, and D = laurtrimonium chloride. H and E stain at 126× magnification.

ceramide spots were not quantified because they could not be separated from the glycosphingolipids.

The precision of the quantitation methodology was estimated from triolein. Ten 20-μg spots of triolein gave an automatic external standard channels ratios (AEC) mean and standard deviation of 0.0984 ± 0.0055. This represents the combined error due to the AEC methodology and the spotting error of the 10-μl-TLC syringe.

Surfactant induced lipid changes. In Table 1, the epidermal lipids that were extracted from the accommodated guinea pigs and the water controls are given. Phosphatidylcholine was the dominant polar lipid, but triglycerides and cholesteryl esters were the most prevalent neutral lipids. Lipid changes due to treatments that were significantly different than control are also indicated. Two types of changes were apparent. For the anionic and nonionic treatments, sphingomyelin, cholesterol and fatty acids were elevated; for the cationic surfactant, phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine were elevated 371%, 170% and 150%, respectively, above control. In addition, for all three treatments, triglycerides were significantly lower than control.

Correlation analysis. Linear regression analysis of the data in Table 1 was carried out between all possible pairs of lipids (see Materials and Methods). These values are tabulated in the correlation Table 2. Although the table is symmetrical about the main diagonal, the lower triangular portion of the table is shown for ease of comparing rows and columns. In addition, to facilitate the comparison of the stronger correlations, numerical values of the correlation were given only when the correlation coefficient was 0.70 or higher. The symbols 0, + and − were used to indicate ranges of correlations below this arbitrary cutoff value.

TABLE 1

Variation in Lipid Composition after Surfactant Accommodation[a]

Lipid	Water control	Surfactant treatment[b]		
		Anionic	Nonionic	Cationic
Polar lipids				
Sphingomyelin	3.2 (0.2)	3.8 (0.2)*	4.5 (0.2)*	3.9 (0.1)
Phosphatidylserine	0.7 (0.2)	0.6 (0.2)	1.1 (0.2)	2.6 (0.5)**
Phosphatidylethanolamine	4.4 (0.3)	3.9 (0.1)	5.5 (0.6)	7.5 (0.3)**
Phosphatidylcholine	10.7 (0.5)	8.2 (0.2)	13.6 (0.7)	16.3 (0.5)**
Neutral lipids				
Cholesterol	6.2 (0.4)	7.4 (0.3)**	8.8 (0.4)**	6.9 (0.2)
Triglyceride	28.7 (0.5)	20.4 (1.1)**	13.7 (0.8)**	18.0 (0.8)**
Cholesteryl esters	39.1 (1.0)	46.4 (1.4)*	42.7 (1.0)	38.8 (1.0)*
Fatty acids	6.9 (0.2)	9.3 (0.4)**	10.2 (0.5)**	5.9 (0.3)*

[a]Wt % (sd).

[b]Anionic surfactant, sodium lauryl sulfate, n = 8; nonionic surfactant, laureth-1, n = 9; cationic surfactant, laurtrimonium chloride, n = 9; and water control, n = 7.

*$p < 0.05$.

**$p < 0.01$.

TABLE 2

Correlation[a] Table for Lipid[b] Changes with Treatment

	S	PS	PE	PC	C	TG	CE	FA
S		+	+	++	+0.93	−0.98	+	++
PS	+		+0.98	+0.90	0	−	−−	−−
PE	+	+0.98		+0.96	0	−−	−−	−−
PC	++	+0.90	+0.96		0	−−	−−	−−
C	+0.93	0	0	0		−0.87	++	+0.82
TG	−0.97	−	−−	−−	−0.87		−	−−
CE	+	−−	−−	−−	++	−		+0.80
FA	++	−−	−−	−−	+0.82	−−	+0.80	

[a]Linear correlation coefficient, r, for two different lipids with respect to their mean lipid compositions in Table 1 (for water control, anionic treatment, nonionic treatment and cationic treatments).

[b]Lipids: S = sphingomyelin, PS = phosphatidylserine, PE = phosphatidylethanolamine, PC = phosphatidylcholine, C = cholesterol, TG = triglycerides, CE = cholesteryl esters, FA = fatty acids.

0, r ranges from 0 to ±0.20.

±, r ranges from ±0.20 to ±0.40.

±±, r ranges from ±0.40 to ±0.70.

An examination of the sphingomyelin row shows that this lipid was positively correlated with all lipids except triglycerides. The very high correlations with respect to cholesterol (+0.93) and the triglycerides (−0.98) indicate, with a high degree of reliability, that as sphingomyelin levels increase, cholesterol levels also increase, but levels of the triglycerides decrease (see Materials and Methods). The cholesterol column shows a similar trend with respect to sphingomyelin and the triglycerides. In addition, a very poor correlation between cholesterol and the phosphatidylethanolamine-based phospholipids is evident.

For the phospholipids, phosphatidylethanolamine shows a very strong correlation with both phosphatidylserine (+0.98) and phosphatidylcholine (+0.96). These latter two lipids also show a strong mutual relationship for each other (+0.90).

Cholesteryl esters and fatty acids show weaker correlations with the other lipids, but interact moderately with one another (+0.80).

DISCUSSION

In this study, it is proposed that predictions can be made regarding the coordinated enzyme synthesis of various biosynthetic components in a given tissue provided that these components can be assayed and that their levels can be altered. Such an analysis can never replace precursor-product tracer studies as an absolute means of understanding enzyme mechanisms. However, we believe it can be used as an initial screen to map out a research strategy in a new tissue. The method of analysis that is proposed is a systematic examination of binary correlation coefficients between all possible pairs of components in the tissue of interest, i.e., a factorialized correlation analysis. The guinea pig epidermis was examined in this study.

When these animals are topically treated with an irritating surfactant at a concentration that is powerful enough to elicit a strong irritation response but weak enough to be sublethal, they are able to somehow adapt to the irritant and enter a clinical phase, under continual surfactant treatment, in which their skin returns visually to a seemingly normal state. McOsker and Beck (12) were the first to describe the histological characteristics of the anionic surfactant accommodation by skin of the guinea pig.

In this study, guinea pigs were chronically treated with lauryl derivatives of anionic, nonionic and cationic surfactants and with water. Thus, four different treatment groups were obtained for lipid analysis. Because the treatments were carried out until an accommodation response was elicited, it was assumed that the lipid biosynthetic apparatus in the skin had adjusted to a new steady-state characteristic of that particular accommodated condition.

We believe two different types of inferences might be gleaned from the factorialized correlation table, Table 2: one regarding established biosynthetic relationships and one regarding unexpected or new relationships. Established relationships concerning sphingomyelin:cholesterol and regarding phosphatidylethanolamine:phosphatidylserine:phosphatidylcholine will now be examined.

Strong correlations were observed between sphingomyelin and cholesterol in the guinea pig epidermis ($r = +0.93$). This appears to parallel observations of sphingomyelin and cholesterol biosynthesis in other tissues. The relationship is related to the fact that, in cells, these two lipids are found exclusively in membranes. Patton found a strong correlation between cholesterol and sphingomyelin in a variety of organelle membranes (13). An increasing gradient of these lipids exists from the nuclear, mitochondrial, endoplasmic reticular and Golgi to the plasma membrane (14,15). There is also some evidence that the coordinated biosynthesis of these lipids is due to a sphingomyelin regulation over cholesterol (16–18).

Regarding the phospholipids, it was found that phosphatidylethanolamine was highly correlated with phosphatidylcholine ($r = +0.96$) and phosphatidylserine ($r = +0.98$). This appears reasonable because phosphatidylethanolamine can serve as a precursor for both of these lipids. Phosphatidylcholine is formed from phosphatidylethanolamine in plasma membranes by the successive action of two different methyltransferases (19,20). As phosphatidylethanolamine is methylated, it moves from the inner leaflet of the membrane to the outer. Thus phosphatidylethanolamine and phosphatidylcholine are only found on the inner and outer leaflets, respectively. Phosphatidylserine, like phosphatidylethanolamine, is found exclusively on the inner leaflet. It can only be synthesized from preformed phospholipids (21)—primarily phosphatidylethanolamine—by the base exchange reaction (22–24). The higher correlation of phosphatidylserine with phosphatidylethanolamine ($r = +0.98$), compared with phosphatidylcholine ($r = +0.90$), may reflect the greater accessibility of the former substrate to the inner leaflet synthetic site.

One of the unexpected lipid changes that occurred in this study regards triglycerides. Triglyceride levels were lower than in the water control for all surfactant treatments (Table 1). At the same time, for the anionic and nonionic treatments, cholesterol, sphingomyelin and fatty acids were elevated. For the cationic treatment, the serine, ethanolamine and choline phospholipids are elevated and fatty acids are lowered. We are not certain why these changes occur.

Because cholesterol and sphingomyelin are highly correlated ($r = +0.93$), one might expect that the strong negative correlation between sphingomyelin and triglycerides ($r = -0.97$) would also imply an equally strong negative correlation between cholesterol and triglycerides. The fact that the actual correlation between these two lipids is somewhat lower ($r = -0.87$) seemed to indicate that one of the four treatments breaks the normal proportional relationships between the lipids. The cationic treatment appears to be such a treatment. When the cationic treatment is omitted from the regression, water, the anionic and the nonionic treatments give nearly a perfect correlation ($r = -0.99$). Similarly, correlations between cholesterol:fatty acids and sphingomyelin:fatty acids improve from $r = +0.82$ to $+0.96$ and from $r = +0.61$ to $+0.96$, respectively. New correlations omitting the cationic treatment from the regression are given in Table 3.

A comparison of the correlation Tables 2 and 3 shows how the influence of the cationic treatment affects the correlations. Because the sphingomyelin:cholesterol correlation now becomes exactly +1.00, Table 3 shows complete symmetry with respect to these two lipids, i.e., the

TABLE 3

Correlation[a] Table for Lipid[b] Changes with Treatment Omitting the Cationic Treatment

	S	PS	PE	PC	C	TG	CE	FA
S		+0.78	++	++	+1.00	−0.99	++	+0.96
PS	+0.78		+0.99	+0.96	+0.78	−0.71	−	++
PE	++	+0.99		+0.99	++	−−	−	++
PC	++	+0.96	+0.99		++	−−	−	+
C	+1.00	+0.78	++	++		−0.99	++	+0.96
TG	−0.99	−0.71	−−	−−	−0.99		−−	−0.98
CE	++	−	−	−	++	−−		+0.70
FA	+0.96	++	++	+	+0.96	−0.98	+0.70	

[a]Linear correlation coefficient, r, for two different lipids with respect to their mean lipid compositions in Table 1 (for water control, anionic treatment and nonionic treatments).

[b]Lipids: S = sphingomyelin, PS = phosphatidylserine, PE = phosphatidylethanolamine, PC = phosphatidylcholine, C = cholesterol, TG = triglycerides, CE = cholesteryl esters, FA = fatty acids.

0, r ranges from 0 to ±0.20.

±, r ranges from ±0.20 to ±0.40.

±±, r ranges from ±0.40 to ±0.70.

S row equals the C column. The symmetry of phosphatidylserine and phosphatidylcholine with phosphatidylethanolamine is also evident. Moreover, the removal of the cationic treatment influence from the regression now reveals the strong negative correlation of triglycerides with fatty acids (r = −0.98). This may reflect a catabolism of epidermal triglycerides to fatty acids or it may relect the fact that the triglycerides are more readily extracted from the cells and are slow to recover their pretreatment levels. Only precursor-tracer studies can elucidate this point.

One of the unexplained reactions to chronic epidermal irritation is the accommodation phenomenon. Suskind has recommended that cellular and biochemical factors should be investigated to explain this "turning off" of the inflammatory response (25). A speculative mechanism that might begin to explain the observed lipid changes is an adaptive response by the cell to reduce surfactant-induced fluidization of plasma membrane lipids (26-29). This could reduce inflammation by decreasing the release of chemotactic factors in L-1- and SLS-accommodation skin.

In summary, factorialized correlation tables provide a systematic method for examining binary relationships between the different lipids in a tissue. Two different types of correlation response patterns emerged: one that agreed with established lipid couplings (sphingomyelin: cholesterol, phosphatidylethanolamine:phosphatidylserine:phosphatidylcholine) and one that reflected new lipid patterns unique to the accommodation process (cholesterol:triglyceride, sphingomyelin:triglyceride). Such a strategy might be useful in two different ways. First, it might be used as a method for quickly estimating whether relationships that have been established in other tissues hold in the tissue of interest. Secondly, it could be used to search for new relationships that have not been previously discovered. Potentially, this approach can be used to improve the efficiency of a research effort by isolating the established from the new. Although, precursor-product relationships between lipids cannot be inferred from these types of procedures, the analysis may provide the basis for defining the most fruitful tracer experiments. These studies could then be carried out in a prioritized manner.

REFERENCES

1. Adams, R.M. (1969) in *Occupational Contact Dermatitis*, p. 6, Lippencott Publishers, New York, NY.
2. Mathias, C.G.T., and Maibach, H.I. (1978) *Clin. Toxicol. 13*, 333-346.
3. Opdyke, D.L., and Burnett, C.M. (1965) *Toilet Goods Assoc. 44*, 3-4.
4. Stanley, J., Foidart, J., Murray, J., Martin, G., and Katz, S. (1980) *J. Invest. Dermatol. 74*, 54-58.
5. Bligh, E., and Dyer, W. (1959) *Can. J. Biochem. Physiol. 37*, 911-917.
6. Shand, J.H., and Noble, R.C. (1980) *Anal. Biochem. 101*, 427-434.
7. Snyder, F., and Moehl, A. (1969) *Anal. Biochem. 28*, 503-509.
8. Radin, N.S., Deshmukh, G.D., Selvam, R., and Hospattankar, A.V. (1982) *Biochim. Biophys. Acta 713*, 474-478.
9. Nobel, R.C., Shand, J.H., and Wagstaff, H.F. (1982) *Anal. Biochem. 122*, 47-51.
10. Nobel, R.C., Shand, J.H., and West, I.G. (1982) *LKB Instr. J. 29*, 1-7.
11. Selvam, R., and Radin, N.S. (1981) *Anal. Biochem. 112*, 338-345.
12. McOsker, D.E., and Beck, L.W. (1967) *J. Invest. Dermatol. 48*, 372-383.
13. Patton, S. (1970) *J. Theor. Biol. 29*, 489-491.
14. Rouser, G., Kritchevsky, G., and Yamamoto, A. (1972) *Adv. Lipid Res. 10*, 261-360.
15. Barenholz, Y. (1984) in *Physiology of Membrane Fluidity* (Shinitzky, M., ed.) Vol. I, pp. 131-173, CRC Press, Boca Raton, FL.
16. Frederickson, D.S., and Sloan, H.R. (1972) in *The Metabolic Basis of Inherited Disease* (Frederickson, D.S., ed.) 3rd ed., pp. 7813-807, McGraw-Hill, New York, NY.
17. Sakuragawa, N., Sakuragawa, M., Kuwabara, T., Pentchev, P.G., Barranger, J.A., and Brady, R.O. (1977) *Science 196*, 317-319.
18. Elias, P.M., Lampe, M.A., Chung, J., and Williams, M.L. (1963) *Lab. Invest. 48*, 565-577.
19. Hirata, F., and Axelrod, J. (1978) *Nature 275*, 219-220.
20. Hirata, F., and Axelrod, J. (1978) *Proc. Natl. Acad. Sci. USA 75*, 2348-2352.
21. Borkenhagen, L.F., Kennedy, E.P., and Fielding, L. (1961) *J. Biol. Chem. 236*, PC28-PC30.
22. Hubscher, G. (1961) *Biochim. Biophys. Acta 57*, 555-561.
23. Dennis, E.A., and Kennedy, E.P. (1972) *J. Lipid Res. 13*, 263-267.
24. Bjerve, K.S. (1973) *Biochim. Biophys. Acta 296*, 549-562.

25. Suskind, R.R. (1977) *Environ. Health Perspec. 20,* 27-37.
26. Hill, M.W., and Bangham, A.D. (1975) *Adv. Exp. Med. Biol. 59,* 1-9.
27. Chin, J.H., Parsons, L.M., and Goldstein, D.B. (1978) *Biochim. Biophys. Acta 513,* 358-363.
28. Chin, J.H., and Goldstein, D.B. (1984) *Lipids 19,* 929-935.
29. Shinitzky, M., and Inbar, M. (1974) *J. Mol. Biol. 85,* 603-615.

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METHODS

Quantification of Oxysterols in Dutch Foods: Egg Products and Mixed Diets

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A sensitive and specific method is described for quantifying various cholesterol oxidation products in foodstuffs, including 7β-hydroxycholesterol, cholesterol-α-epoxide, cholestane-triol, 7-ketocholesterol and 25-hydroxycholesterol. A chloroform-methanol extract of the food was fractionated over two successive silica columns. Two fractions containing different classes of oxysterols were then analyzed as trimethylsilyl derivatives by capillary gas liquid chromatography, using on-column injection and a temperature gradient from 70 to 200°C. The detection limit was about 0.5 μg/g dry weight for egg yolk powder. Fresh egg yolk contained only 1.2 μg/g of total oxides per g dry weight, showing that artifactual oxidation during the procedure was minimal. Recovery of 5 pure oxysterols added to egg yolk at levels of 6.5 and 10 μg/g was between 93 and 102%. In commercial egg yolk and whole egg powder stored for one year, total amounts of oxysterols ranging from 21 to 137 μg/g dry weight were found. In duplicates of mixed Dutch diets, total amounts ranged from 3.6 to 6.2 μg/g dry weight. Duplicates containing mostly fried and baked foods did not have higher levels than duplicates in which foods had been prepared by boiling or left raw. We conclude that a normal mixed diet provides only minor amounts of cholesterol oxidation products.
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Cholesterol oxidizes spontaneously in air, yielding a variety of oxidation products. At least 30 oxidation products of cholesterol have been reported (1). Many of these have potent biological effects (2). Some are cytotoxic and angiotoxic and may play a role in atherogenesis (3). Certain oxysterols may also be carcinogenic or mutagenic (4–6).

Foods containing cholesterol, particularly those that have been exposed to heat and air during processing or have been stored at ambient temperature, might contain autoxidation products of cholesterol. However, little information is available on the actual presence of cholesterol oxidation products in foods. In egg products, cholestanetriol (7), 7-hydroxycholesterol isomers (8,9) and cholesterol-α-epoxide (10) have been found, but often only after irradiation with UV light. A recent paper (11) reported that no oxysterols could be detected in fresh egg yolk. Spray-dried egg yolk powder contained traces of oxysterols when fresh or stored for 2 months at 4°C, but prolonged storage gave lipids extracts that contained variable levels (0–12 ppm) of various oxidation products. Variable amounts of cholesterol oxidation products have also been found in lard (12), in anhydrous milk fat and nonfat dry milk stored for 2 years at ambient temperature (13) and in heated beef tallow (14,15).

This paper describes a method for the quantitative determination of some cholesterol oxidation products in foods. It employs an isolation and prefractionation step using silica column chromatography. Oxysterols from food products containing high amounts of lipids and a relatively low cholesterol level can thus be purified and enriched in an adequate way before capillary gas liquid chromatography. The levels of some cholesterol oxidation products in fresh egg yolk, dehydrated egg and milk products stored for various periods of time, and in some mixed Dutch diets were estimated with this method.

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Overview. The food is extracted with chloroform-methanol, and lipids other than sterols are removed by column chromatography on silica column I. The sterol fraction is then applied to silica column II, which serves to separate cholesterol (usually present to excess) from oxysterols. The latter are collected in two fractions of different polarity, each of which is then analyzed by capillary gas chromatography. Analysis of two samples in duplicate, plus appropriate standards and controls, takes one technician about two days.

Food samples. Whole egg powder, egg yolk powder, desugared whole egg powder and desugared egg yolk powder were obtained from four different companies in the Netherlands. Because not all companies could provide all four products, the total number of samples analyzed was only 11. Samples were stored in polyethylene bags in a refrigerator at 4°C for approximately one year. In addition to these products, an egg yolk powder stored for four years at ambient temperature was also available. A subsample of this egg yolk powder was irradiated with UV light (254 nm) with a Camag universal UV-lamp (type TL-900) at a distance of 10 cm for three weeks. During this period the powder was mixed with a spatula once a day.

Two samples of dry full-fat milk stored for two and seven years, and three mixed human diets prepared for a study on the carcinogenicity of different diets in rats were also analyzed for cholesterol oxidation products. This latter study aimed at determining the effects on tumor incidence of baking, frying and grilling, and of adding extra fruits and vegetables to the diet. The diets were duplicates of the average diet consumed in The Netherlands. They were kitchen-prepared in batches of several hundred kg and then freeze-dried and pelleted. For the first diet, all foods were left raw; for the second diet,

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Abbreviations: Cholestane-triol, cholestan-3,5,6-triol (3β, 5α, 6β)-; cholestanol, cholestan-3-ol (5α, 3β)-; cholesterol-α-epoxide, cholestan-3-ol,5,6-epoxy- (3β,5α, 6α)-; cholesterol-β-epoxide, cholestan-3-ol,5,6-epoxy- (3β,5β,6β)-; 7α-hydroxycholesterol, cholest-5-en-3,7-diol (3β,7α)-; 7β-hydroxycholesterol, cholest-5-en-3,7-diol (3β,7β)-; 20-hydroxycholesterol, cholest-5-en-3,20-diol (3β)-; 25-hydroxycholesterol, cholest-5-en-3,25-diol (3β)-; 7-ketocholesterol, cholest-5-en-3-ol-7-one (3β)-.

products were fried, baked or grilled when appropriate. The third diet was equal in composition to the second one, but contained added fruits and vegetables of supposedly anticarcinogenic action. A commercial pelleted rat chow was also analyzed.

Solvents, reagents and column materials. Chloroform (99.5%, v/v) from Janssen Chimica (Brussels, Belgium) (No. 15.821.10) with 0.75% (v/v) ethanol was used for chromatography on column I (Fig. 1). For the fractionation on column II, HPLC grade chloroform stabilized with 2-methyl-2-butene was used from Merck (Darmstad, West Germany) (no. 2444), as were methanol (No. 6009), and acetone (No. 14). Hexane (No. RH 1002) was obtained from Rathburn (Walkerburn, Scotland). Silylation reagents, hexamethyldisilazane (No. 84770) and trimethylchlorosilane (No. 88530) were from Pierce Eurochemie (Oud-Beijerland, The Netherlands) and dried pyridine (No. 7463) was from Merck. The purity of all solvents was checked by chromatography and found to be satisfactory.

Silica gel 60 (Merck) (No. 7734) of particle size 63–200 μm was used for column I. It was purified and activated by suspending 500 g in 1 liter 3 M HCl for 60 min and then washed with deionized water until neutral, dried overnight at 110–120°C and stored in a polyethylene bottle.

For column II, silica gel for low-pressure column chromatography from Baker Chemical B.V. (Deventer, The Netherlands) (No. 7024-1) was used as purchased. The average particle size was 40 μm (30–60 μm) and the average pore diameter 6 nm. Cholestane-triol (No. 4700), 7β-hydroxycholesterol (No. 6430), 7α-hydroxycholesterol (No. 6420), cholesterol-α-epoxide (No. 4130), 7-ketocholesterol (No. 6970) and 25-hydroxycholesterol (No. 6510) were from Steraloids Inc., Wilton, NH; 5α-cholestane (No. 17060) was from Pfaltz and Bauer, Inc., Stanford, CT; and betulin (No. B-9757) was from Sigma Chemical Co., St. Louis, MO.

Saponification. Saponification followed by extraction of the unsaponifiable fraction has often been used in the determination of sterols in foods (16–18). It has been reported (10), however, that up to 75% of the cholesterol-α-epoxide was lost during saponification. Therefore we subjected a cholesterol sample dating back to 1963, a UV-irradiated egg yolk powder and a mixture of pure oxysterols to two degrees of saponification. The oxysterol mixture contained 7-ketocholesterol, 25-hydroxycholesterol and cholestane-triol. Mild saponification with 100 ml 2 M ethanolic NaOH per l to 2 g of each sample was carried out at ambient temperature for one night, and normal saponification was done under reflux in a boiling water bath for 30 min under conditions, as have been reported elsewhere (19).

Extraction of lipids. An aliquot of dry sample containing 1–1.5 g lipids was transferred to a 150 ml wide-mouth conical flask and 100 ml Folch reagent (chloroform/methanol, 2:1, v/v) was added. The mixture was then homogenized with a Polytron Model PT 10/35 homogenizer (Kinematica, GmbH, Lucerne, Switzerland) at speed 5 for about 15 sec. After 10 min of equilibration, the extract was filtered through a defatted folded filter paper (MN 615¼, Macherey-Nagel, Düren, Germany). The residue was washed three times with Folch reagent and the combined filtrate was dried under vacuum with a rotary film evaporator. The vacuum of the evaporator was released with nitrogen gas. The lipids were redissolved in 5 ml of chloroform.

Silica gel column chromatography. For the isolation of cholesterol and oxysterols, a glass column (length 150 mm, o.d. 40 mm) equipped with a teflon stopcock and connected by a glass joint to a glass solvent reservoir was used (column I). The column was packed using a slurry of 50 g activated and purified Silica Gel 60 in chloroform. After settling of the adsorbent, the bed was vibrated with a hand vibrator to remove remaining air bubbles. Then the silica gel was washed with 75 ml chloroform, at a flow rate of 2–3 ml/min. The lipid extracted was transferred to the column, and two portions of 5 ml of chloroform were used for rinsing the flask and then added to the column.

The neutral lipids were eluted with 175 ml of chloroform and the sterols and their oxidation products were then eluted with 150 ml of acetone. The phospholipids remained on the column. The acetone eluent was collected into a flat-bottom extraction flask and evaporated to dryness under vacuum with a rotary evaporator. The vacuum of the evaporator was released with nitrogen gas and the residue was redissolved immediately in a 3-ml chloroform-acetone mixture (98:2, v/v).

For the prefractionation of oxysterols, a 10 g silica gel column (column II) was prepared as follows. In a separation funnel, 10 g of silica for flash chromatography was mixed with chloroform acetone (98:2, v/v) to form a slurry and was left at room temperature for 15 min. Then it was shaken again and the slurry poured into a glass column of length 250 mm and o.d. 13 mm, which was connected by a ball joint to a solvent reservoir. The column contained a small plug of glass wool covered by a thin layer of sea sand in chloroform-acetone mixture (98:2, v/v) on top of the teflon stopcock at the bottom of the tube. After settling of the adsorbent, the column was vibrated with a hand vibrator and rinsed with 50 ml chloroform-acetone solvent mixture (98:2, v/v). A flow rate of 2–3 ml/min was maintained by a nitrogen pressure of about 50 kPa on the solvent reservoir. The solvent level was allowed to descend to the top of the bed, and the sterols were layered on top of the adsorbent, using 3 ml rinsing aliquots of the chloroform-acetone (98:2, v/v) mixture. The column was then successively eluted with 100 ml of three different chloroform-acetone mixtures: A (98:2, v/v), B (80:20, v/v) and C (50:50, v/v).

Cholesterol eluted in fraction A. So did cholestanol and, therefore, quantification of this compound was not possible. Cholesterol-α-epoxide, 7-ketocholesterol, 25-hydroxycholesterol and the major part of the 7β-hydroxycholesterol eluted in fraction B. The cholestane-triol and a minor part of 7β-hydroxycholesterol eluted in fraction C. Common plant sterols eluted in the cholesterol fraction (fraction A) and, thus, did not interfere with the later gas-chromatographic separation of oxysterols (data not shown).

Fraction A was discarded, and fractions B and C were collected separately in extraction flasks. Then 1 ml of an internal standard solution containing 20 μg of each standard component (5α-cholestane and betulin) was added to both fractions. The solvents were evaporated under vacuum with a rotary thin-film evaporator. The vacuum was released with nitrogen gas, and the residues were redissolved in chloroform and transferred to a 1 ml conical vial for derivatization.

Derivatization. The residue was dried under nitrogen. Then 0.5 ml of pyridine hexamethyldisilazane trichloromethylsilane (10:2:1, v/v) was added. The vial was closed with a screw top sealed with teflon. It was left to stand for 30 min at ambient temperature. After removal of the excess of pyridine under a stream of nitrogen, 400 μl of hexane was added. The vial was shaken and centrifuged, and 0.5 μl of the hexane solution was injected into the gas chromatograph.

Gas liquid chromatography. A Packard Instruments (Delft, The Netherlands) gas liquid chromatograph Model 433 with a flame ionization detector, a digital integrator and a capillary on-column injector from Chrompack (Middelburg, The Netherlands) was used. It was equipped with a 25 m × 0.22 mm fused silica capillary WCOT column CP Sil5CB (Chrompack), with a film thickness of 0.12 μm and a coating efficiency of about 90%. The oven temperature program was: initial temperature 70°C for 2 min followed by a rise to 200°C at a rate of 15°C/min and, then, by another rise at a rate of 10°C/min to an upper temperature of 295°C. The oven was held at this upper temperature for various lengths of time until all components were eluted. Other conditions were: carrier gas, hydrogen; pressure, 150 kPa; make-up gas, nitrogen with flow rate 15/min; and detector temperature, 325°C.

Prior to the analysis of samples, the system was calibrated with a mixture of pure compounds, and response factors relative to the average response of betulin and 5α-cholestane were calculated. If the betulin or 5α-cholestane peaks could not be identified due to interference from other unknown compounds, then response factors were calculated relative to only one of these compounds.

Mass spectrometry. To confirm the tentative gas chromatographic identification, some samples were also analyzed on a Finnigan-MAT 8200 mass spectrometer (Finnigan Corp., Cincinnati, OH) equipped with a Finnigan-MAT ss200 data system.

A Varian 3700 gas chromatograph (Varian Associates Inc., Sunnyvale, CA) fitted with a 25 m × 0.5 mm i.d. CP-Sil5 glass capillary column (Chrompack, 4330 EW Middelburg, The Netherlands) was attached to the mass spectrometer through an open split interface. Helium was used as carrier gas at a flow rate of 6 ml/min. The initial oven temperature was 200°C and was increased to 320°C at a rate of 40°C/min. The injector and interface temperatures were 250°C.

Spectra were obtained by electron impact ionization within a mass range of 20–700 m/e. The scan speed was 1 sec/decade. Background subtraction and renormalization to the most intense peak were performed. Peaks were identified by comparing their mass spectra with those of the pure compounds.

RESULTS AND DISCUSSION

Effect of saponification. The results of normal and mild saponification of the old cholesterol sample, the UV-irradiated egg yolk powder and the mixture of pure oxysterols showed that 7-ketocholesterol is very sensitive to alkaline hydrolysis. Only 11–40% of the original amount was recovered under mild saponification conditions, but saponification at 100°C caused a total disappearance of 7-ketocholesterol. 25-Hydroxycholesterol was not very sensitive to alkaline hydrolysis (recovery range, 89–121%), while cholestane-triol was somewhat sensitive to both mild and normal saponification (recovery range, 78–90%). Tsai et al. (17) reported that up to 75% of the cholesterol-α-epoxide was lost during saponification. Therefore, no saponification was used. The error introduced by not saponifying is probably small, as only some 10% of the cholesterol in foods is esterified (20).

Silica chromatography. Silica gel column I served to remove the nonsterol lipids. From an egg yolk powder containing 62.5% of weight as lipid, 79.7% of these lipids were recovered in the chloroform fraction, and the acetone fraction contained 3.7% of the lipids and 80–90% of the total amount of cholesterol.

Column II was used to remove the bulk of cholesterol and to prefractionate the oxidation products. For samples with a highly complex composition such as egg yolk such prefractionation proved necessary, as certain oxysterols could not be separated by only gas chromatography.

Gas liquid chromatography. In Figures 1 and 2 a gas chromatogram of an egg yolk sample is shown. In between the two internal standard peaks, 5α-cholestane and betulin, a large number of peaks was present. Various constituents were first identified tentatively by their retention time relative to 5α-cholestane and betulin. The retention times and response factors are given in Table 1. The mean standard deviation of 0.009 for response factors relative to betulin is smaller than the standard deviation of 0.027 found for response factors relative to 5α-cholestane, because for some samples, the 5α-cholestane was not separated well from other unknown components. The separation of 7-ketocholesterol and 25-hydroxycholesterol was also critical. Due to changes of the capillary column with time, these two components sometimes could no longer be separated after the column had been in use for a prolonged period. The problem could be solved by inserting a 5-min halt at 270°C into the final part of the temperature program.

As for oxysterols not listed in Table 1, 20-hydroxycholesterol was never detected in foods and was therefore not studied extensively. We were unable to quantitate 7α-hydroxycholesterol, because this peak partially coincided with that of cholesterol. In addition, the number of oxysterols that we could quantitate was limited to those for which pure standards were available. Therefore, we could identify the 5,6α-, but not the 5,6β-isomer of cholesterol

TABLE 1

Retention Times of Various Cholesterol Oxidation Products Upon GLC and Response Factors[a]

Cholesterol oxidation product	Retention time	Relative response factor[a]	
		5α-cholestane	Betulin
5α-Cholestane	19.80	1.000	–
7β-Hydroxycholesterol	23.33	0.926 ± 0.022	0.906 ± 0.005
Cholesterol-α-epoxide	23.48	1.234 ± 0.037	1.207 ± 0.015
Cholestane-triol	26.90	1.074 ± 0.026	1.038 ± 0.013
7-Ketocholesterol	25.15	1.100 ± 0.025	1.077 ± 0.005
25-Hydroxycholesterol	25.50	0.931 ± 0.025	0.911 ± 0.008
Betulin	27.50	–	1.000

[a]The relative response factor equals (concentration of component/peak area of component)/(concentration of internal standard/peak area of standard); mean ± SD of 3 measurements is given. Factors are relative to the two internal standards, 5α-cholestane and betulin.

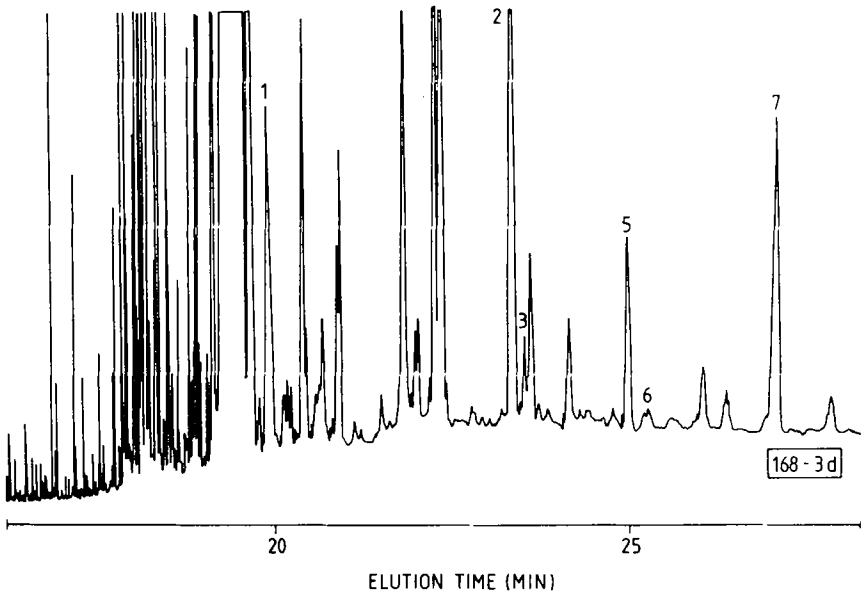

FIG. 1. Gas chromatogram of cholesterol oxidation products in egg yolk powder: fraction B, containing the less polar oxysterols. 1, 5α-cholestane (internal standard); 2, 7β-hydroxycholesterol; 3, cholesterol-α-epoxide; 5, 7-ketocholesterol; 6, 25-hydroxycholesterol; 7, betulin (internal standard).

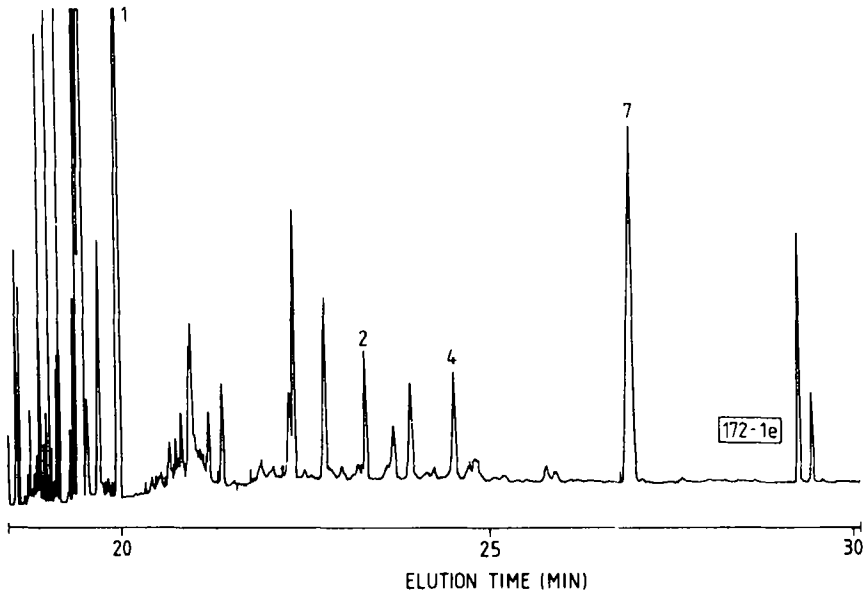

FIG. 2. Gas chromatogram of cholesterol oxidation products in egg yolk powder: fraction C, containing the more polar oxysterols. 1, 5α-cholestane (internal standard); 2, 7β-hydroxycholesterol; 4, cholestane-triol; 7, betulin (internal standard).

epoxide. As the gas chromatography column separated mainly by boiling point, there is a possibility that the α and the β isomer of cholesterol epoxide eluted as one peak. Alternatively, the unidentified peaks in the oxysterol region of the chromatogram (Figs. 1 and 2) could contain this or other unidentified oxysterols.

The stability of the various oxidation products on the column was very good, as indicated by the low standard deviations of the relative response factors. Low standard deviations were obtained only if the sample was injected directly on the column at a temperature of 70°C. If we used a more conventional splitless flash heater at 280–300°C for sample, injection we found large standard deviations, possibly caused by variable decomposition of oxysterols in the flash heater (data not shown).

Recovery, precision and detection limit. A standard mixture containing 20 μg each of cholesterol-α-epoxide, cholestane-triol, 25-hydroxycholesterol and 200 μg of cholesterol

was taken through the full procedure. Recoveries were between 86 and 98% (Table 2). The same oxysterols were added in known amounts (6.5 and 10 μg/g) to egg yolk powder. Recoveries (Table 2) varied between 93 and 98% at the 6.5 μg/g level and between 94 and 102% at the 10 μg/g level.

The reproducibility for independently worked-up samples is given in Table 3. The coefficient of variation tended to be lower, the higher the level of the analyte. For 25-hydroxycholesterol, a concentration of 0.3 μg/g with a coefficient of variation of 72% was found. Thus, this is about the lowest level of oxysterol that can be detected with our method in samples with highly complex matrices such as egg yolk.

Identification. Both the gas chromatographic separation and the detector response were poorer for the GC–MS analyses than for the routine gas chromatographic analyses. The use of splitless on-column injection in the latter was probably a major factor in providing a high yield and good separation.

The m/e values of typical fragments in the mass spectra of pure compounds and of oxysterol fractions from egg yolk powder were compared with published data (1). This provided identification of the trimethylsilylether of cholesterol-α-epoxide (m/e 474, 456 and 384), of cholestane-triol (m/e 546, 456, 403, 367 and 321) and of a 7-hydroxycholesterol (m/e 546, 456, 233 and 208); the geometry of the latter (7α or 7β) could not be determined. The quality of the GC–MS analyses proved insufficient to confirm or reject the identifications of the other oxysterols in egg yolk powder.

Levels of oxysterols in foods. Table 4 gives the levels of oxidation products in egg yolk and whole egg powder, and in dry milk. The very low content of oxysterols in fresh egg yolk indicates that artifactual oxidation of cholesterol during sample work up was minimal; the oxysterol concentration of 1 μg/g should be contrasted with the total cholesterol content of about 12,000 μg present in 1 g of egg yolk. A similar result was found by Nourooz-Zadeh and Appelqvist (11). The levels of 7β-hydroxycholesterol (12.8 to 78.0 μg/g) and cholesterol-α-epoxide (4.2 to 46.0 μg/g) found by us in nonirradiated samples of egg yolk and whole egg powder stored one to four years prior to analysis were higher than those found by Nourooz-Zadeh and Appelqvist (11) who found concentrations between 0 and 9.8 μg/g (7β-hydroxycholesterol) and between 0 and 2.5 μg/g (cholesterol-α-epoxide) in samples stored up to one and a half years, and concentrations of 46.8 μg/g (7β-hydroxycholesterol) and of 9.4 μg/g (cholesterol-α-epoxide) in a sample stored for eight years. The levels of cholestane-triol, 7-ketocholesterol and 25-hydroxycholesterol found by us in egg yolk and whole egg powder were generally low (below 15 μg/g), although these too were higher than those of Nourooz-Zadeh and Appelqvist (11). The effect of storage on the concentrations

TABLE 2

Recovery of Cholesterol Oxidation Products From a Mixture of Pure Compounds and From Egg Yolk Powder[a]

Cholesterol oxidation product	Mean percentage recovery		
	Standard compounds	Added to egg yolk powder	
	20 μg each (n = 5)[b]	6.5 μg/g (n = 2)[c]	10 μ/g (n = 4)[b]
7β-Hydroxycholesterol	86 ± 12	95 (85–105)	102 ± 7
Cholesterol-α-epoxide	92 ± 6	98 (92–103)	99 ± 6
Cholestane-triol	90 ± 5	95 (91–99)	94 ± 3
7-Ketocholesterol	98 ± 9	98 (95–101)	96 ± 4
25-Hydroxycholesterol	94 ± 7	93 (92–94)	97 ± 1

[a]To which pure compounds had been added at two different levels. The samples underwent the entire extraction and column chromatography procedure prior to gas chromatography. The standard mixture also contained 200 μg cholesterol.

[b]Mean ± SD.

[c]Mean (range).

TABLE 3

Reproducibility of the Determination of Cholesterol Oxidation Products in Commercial Whole Egg and Egg Yolk Powder

Cholesterol oxidation product	n	Mean (range) (μg/g)	SD (μg/g)	Coefficient of variation (%)
7β-Hydroxycholesterol	12	36.8 (11.9–78.0)	2.18	5.9
Cholesterol-α-epoxide	11	4.4 (1.8–11.2)	0.75	17.0[a]
Cholestane-triol	12	2.8 (0.3–13.9)	0.48	17.2
7-Ketocholesterol	12	16.9 (4.2–46.0)	1.31	7.7
25-Hydroxycholesterol	12	0.3 (0.0–0.5)	0.21	71.8

[a]If one outlier was excluded, the coefficient of variation was 8.6%.

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TABLE 4

Concentration of Cholesterol Oxidation Products in Foods and in Duplicate Diets[a]

Commodity	7-Hydroxycholesterol	Cholesterol-α-epoxide	Cholestane-triol	7-Ketocholesterol	25-Hydroxycholesterol
			(μg/g as is)		
Fresh egg yolk	0.3	0.7	0.0	0.2	0.0
Egg yolk powder					
Manufacturer 1	12.8	5.8	0.3	1.8	0.3
Manufacturer 2	45.1	26.3	0.4	6.5	2.6
Manufacturer 2[b]	74.1	46.0	0.5	9.4	7.3
Manufacturer 3	16.9	8.1	0.0	2.8	1.6
Manufacturer 3[b]	16.8	6.7	0.1	1.8	1.0
Manufacturer 4	22.3	9.7	0.5	3.4	0.5
Manufacturer 4[c]	78.0	25.2	0.5	4.2	13.9
Manufacturer 4[d]	507	2522	62	200	860
Whole egg powder					
Manufacturer 1	21.2	6.4	0.0	3.2	0.4
Manufacturer 2	64.9	35.8	0.5	11.2	2.3
Manufacturer 2[b]	36.8	19.4	0.2	8.1	1.4
Manufacturer 4[b]	40.8	9.6	0.4	3.6	1.9
Manufacturer 4[c]	11.9	4.2	0.0	1.9	0.3
Dry full-fat milk					
Manufacturer 1[e]	2.9	1.2	0.3	0.5	0.8
Manufacturer 2[f]	3.9	4.1	<0.1	1.5	0.1
Commercial rat feed	0.3	0.04	0.0	0.3	0.0
Commercial rat feed with extra fruit and vegetables	0.1	0.02	0.0	0.2	0.0
Duplicate Dutch diet, raw	2.3	0.7	0.1	1.1	0.0
Duplicate Dutch diet, baked/fried/grilled	1.7	0.8	0.1	1.0	0.0
Duplicate Dutch diet, baked/fried/grilled, plus extra fruit and vegetables	2.2	1.7	<0.1	2.0	0.2

[a]Duplicate diets had been freeze-dried and pelleted and stored for one year at 4°C, unless indicated otherwise.

[b]Desugared.

[c]After storage at ambient temperature for 4 years.

[d]After irradiation of sample c with UV light for three weeks.

[e]After 7 years of storage.

[f]After 2 years of storage.

of oxysterols was unclear. In egg yolk powder the concentrations increased upon long-term storage, but in whole egg powder they decreased. It might be that differences in storage temperature at the manufacturers are responsible for this (11). UV-irradiation considerably increased the concentrations of all oxysterols studied.

The total concentration in dry full-fat milk stored between two and seven years did not exceed 10 μg/g. Levels of cholesterol oxidation products in commercial rat chow were also very low (Table 4).

In duplicates of the average diet eaten in the Netherlands, oxysterol levels ranged from 0.0–0.2 μg/g for 25-hydroxycholesterol to about 2 μg/g for 7β-hydroxycholesterol. The levels were not higher in duplicate diets made up of fried, baked and grilled products than in duplicate diets made up of the equivalent raw foods. Thus, the amount of cholesterol oxidized during the frying, baking or grilling of foods appears to be very small. As the daily food intake for an average person is about 500 g, the average Dutchman will ingest about 1 mg of 7β-hydroxycholesterol and 0.5 mg of cholesterol-α-epoxide per day. The diets to which fruits and vegetables of supposedly anticarcinogenic activity had been added, contained somewhat higher levels of 7-ketocholesterol (2.0 μg/g compared with 1.1 and 1.0 μg/g for the other two mixed diets without the extra fruits and vegetables added). In view of the freeze drying and pelleting that our samples had undergone these figures may be considered upper limits. On the other hand, the chromatograms of these duplicate diets did contain quite a few peaks that escaped identification, and one could theorize that normal foods might contain appreciable amounts of oxysterols other than those identified. However, the oxysterols studied by us are those naturally formed from cholesterol under a range of conditions, and it seems unlikely that the reaction of oxygen with cholesterol in foods would produce an entirely different range of products. Therefore we suggest that the most plausible load of oxysterols in the average Dutch diet is at most a few mg per day. Whether such amounts contribute to the development of chronic diseases is at present not known.

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REFERENCES

1. Smith, L.L. (1981) *Cholesterol Autoxidation*, Plenum Press, New York, NY.
2. Smith, L.L. (1987) *Chem. Phys. Lipids 44*, 87–125.
3. Peng, S.K., and Taylor, C.B. (1984) *World Rev. Nutr. Diet. 44*, 117–154.
4. Lo, W.B., and Black, H.S. (1973) *Nature 246*, 489–491.
5. Ames, B.N. (1984) *Science 224*, 668–760.
6. Sevanian, A., and Peterson, A.R. (1986) *Food Chem. Toxic. 24*, 1103–1110.
7. Fieser, L.F., and Bhattacharyya, B.T. (1953) *J. Am. Chem. Soc. 75*, 4418–4423.
8. Acker, L., and Greve, H. (1963) *Fette Seifen Anstrichm. 65*, 1009–1012.
9. Chicoye, E., Powrie, W.D., and Fennema, O. (1968) *J. Food Sci. 33*, 581–587.
10. Tsai, L.S., Ijichi, K., Hudson, C.A., and Meehan, J.J. (1980) *Lipids 15*, 124–128.
11. Nourooz-Zadeh, J., and Appelqvist, L.A. (1987) *J. Food Sci. 52*, 57–62 and 67.
12. Williams, L.D., and Pearson, A.M. (1965) *J. Agric. Food Chem. 13*, 573–577.
13. Flanagan, V.P., Ferretti, A., Schwartz, D.P., and Ruth, J.M. (1975) *J. Lipid Res. 16*, 97–104.
14. Won Park, S., and Addis, P.B. (1986) *J. Agric. Food Chem. 34*, 653–659.
15. Bascoul, J., Domergue, N., Olle, M., and Crastes de Paulet, A. (1986) *Lipids 21*, 383–387.
16. Punwar, J.K., and Derse, P.H. (1978) *J. Assoc. Off. Anal. Chem. 61*, 727–730.
17. Kovacs, M.J.P., Anderson, W.F., and Ackman, R.G. (1979) *J. Food Sci. 44*, 1299–1306.
18. Itoh, T., Tamura, T., and T. Matsumoto, T. (1973) *J. Am. Oil Chem. Soc. 50*, 122–125.
19. Van de Bovenkamp, P., and Katan, M.B. (1981) *J. Food Sci. 46*, 291.
20. Sweeney, J.P., and Weihrauch, J.L. (1976) *Crit. Rev. Food Sci. Nutr. 8*, 131–159.

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COMMUNICATIONS

Characterization of 12-Methyl-*cis*-4-tetradecenoic Acid From *Corynebacterium sepedonicum*

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A previously uncharacterized fatty acid found in *Corynebacterium sepedonicum* was analyzed by gas chromatography-mass spectrometry and infrared spectrometry and determined to be 12-methyl-*cis*-4-tetradecenoic acid. This fatty acid represents nearly 6% of the total fatty acids of *C. sepedonicum*, but it is present at significantly lower levels in other *Corynebacterium* species. Therefore, 12-methyl-*cis*-4-tetradecenoic acid provides a useful marker in distinguishing *C. sepedonicum* from other microorganisms.

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Fatty acid analysis of cellular components has been used extensively for the determination of taxonomic relationships and for the rapid identification of medically and clinically important bacteria (1,2). However, few examples of fatty acid analyses of plant pathogenic bacteria have been reported (3,4). Some preliminary studies have been performed in our laboratory involving the use of fatty acid analysis for the identification and differentiation of plant pathogenic *Corynebacteria* (4). During these studies, a fatty acid was detected in saponified extracts of *Corynebacterium sepedonicum* (Spieck. and Kotth.) Skapt. and Burkh. that was previously unidentified. In this paper, we report on the characterization of the unknown fatty acid, and have identified it as 12-methyl-*cis*-4-tetradecenoic acid. The fatty acid was a significant component (>5%) of *C. sepedonicum*, but constituted substantially less in all other plant pathogenic *Corynebacteria* tested (4).

MATERIALS AND METHODS

Bacterial strains were obtained from the American Type Culture Collection (Rockville, MD), Plant Disease Division Culture Collection (Auckland, New Zealand), from individual researchers in the U.S. and Canada, and from isolates recovered by us in our laboratory from samples brought in by area potato growers. Identities of all strains were confirmed by biochemical and physiological methods (5) and by indirect immunofluorescent antibody staining according to De Boer and Weiczorek (6).

Bacterial cultures were harvested according to Suzuki and Komagata (7), and the methyl esters prepared (8). Briefly, the cells were centrifuged and washed twice under aseptic conditions. After lyophilization, 5 mg of bacteria was saponified with 1 ml of 3.75 N NaOH in 25% aqueous methanol at 100°C for 30 min in a water bath, with occasional mixing. The mixture was methylated by adding 2 ml of 3.25 N HCl in 45% methanol and heating for 10 min at 80°C. The fatty acid methyl esters were extracted with 1.25 ml of hexane/diethyl ether (1:1, v/v). The organic phase was washed once with 3 ml of 0.3 N NaOH.

Pyrrolidide derivatives were prepared by the method of Andersson and Holman (9). Hydrogenation was accomplished by adding a small amount of platinum oxide catalyst (ca. 5 mg) to a solution of the fatty acid methyl ester mixture in 2 ml of methanol, then directing a stream of hydrogen gas through the solution for 5 min. Fatty acid methyl esters were separated by thin layer chromatography using a Whatman $LKC_{18}F$ thin layer chromatography plate. The solvent was acetonitrile/water (95:5, v/v). Fatty acid methyl ester components were visualized by exposure of a portion of the plate to iodine. Fatty acid composition was determined with a Hewlett-Packard model 5790 gas chromatograph equipped with a 30 m × 0.25 mm i.d. SPB-1 methylsilicone capillary column (Supelco, Bellefonte, PA). The temperature was programmed from 150°C to 250°C at 4°C/min. Mass spectra were obtained with a Hewlett-Packard model 5992 gas chromatography–mass spectrometry (GC–MS) equipped with a 30 m × 0.22 mm i.d. methylsilicone capillary column. Infrared spectra were obtained with a Nicolet 5-MX Fourier transform spectrometer. Spectra were determined on the sample prepared in a KBr pellet.

Most fatty acids could be identified by comparing the equivalent chain lengths (ECL) of their methyl esters with those of a fatty acid methyl ester standard (Supelco 4-7080, Bacterial Acid Methyl Ester Mix CP). Their identifications were further confirmed from their molecular weights which were determined by GC–MS. Fatty acid methyl esters not identified by this method were converted to pyrrolidide derivatives and analyzed by GC–MS to determine the location of unsaturation and branching.

RESULTS AND DISCUSSION

The acyl components of most plant and animal glycerolipids are usually composed of saturated or unsaturated, straight-chain fatty acids. In contrast with plants and animals, certain bacteria such as *Bacillus* (10) and *Staphylococcus* (11) utilize iso and anteiso methyl-branched fatty acids as the predominant acyl constituents of glycerolipids. The genus *Corynebacterium*, however, differs from *Bacillus* and *Staphylococcus* in that it is heterogenous with respect to fatty acid types. *C. sepedonicum* of this genus belongs to the type II group, which is composed predominantly of iso and anteiso branched-chain fatty acids (7).

In our studies of the fatty acid composition of *C. sepedonicum*, we observed that extracts of the bacteria contained five fatty acids that were each present in

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Abbreviations: ECL, equivalent chain length; GC–MS, gas chromatography–mass spectrometry.

TABLE 1

Mean Percentage, Molecular Weight and Equivalent Chain Lengths of Fatty Acid Methyl Esters Derived From *Corynebacterium sepedonicum*

Fatty acid	Mean % ± SD[a]	Mol wt[b]	ECL[c]
12-Methyl-*cis*-4-tetradecenoic acid	5.9 ± 1.8	254	14.47
12-Methyl-tetradecanoic acid	39.9 ± 2.3	256	14.71
14-Methyl-pentadecanoic acid	15.8 ± 3.1	270	15.64
Hexadecanoic acid	2.8 ± 1.1	270	16.00
14-Methyl-hexadecanoic acid	31.4 ± 5.5	284	16.72

[a]SD, standard deviation; 40 strains of *C. sepedonicum* were analyzed.

[b]Molecular weight determined by GC-MS.

[c]Equivalent chain length of methyl ester on an SPB-1 methylsilicone capillary column.

amounts greater than 2% of the total fatty acid content (Table 1). These five fatty acids constituted >95% of the total fatty acids present. Of these five major fatty acids, the latter four were tentatively identified by comparison of the ECL of their methyl esters with those of a standard. These fatty acids have previously been characterized as components of coryneform bacteria by Bousfield et al. (12) and Suzuki and Komagata (7). We further confirmed their identities by the determination of their molecular weights by GC-MS.

The unidentified fatty acid methyl ester (ECL, 14.47) had a molecular weight of 254 by GC-MS analysis, indicating that it contained 15 carbons with either one double bond or a ring structure. Other characteristic fragments were present at *m/z* 225 $[M - CH_3CH_2]^+$, 222 $[M - CH_3OH]^+$ and 193 $[225 - CH_3OH]^+$. When the methyl ester of this fatty acid was isolated by reversed phase thin layer chromatography and then hydrogenated, the resulting product matched the retention time of 12-methyl-tetradecanoic acid, and its molecular weight was increased by 2 amu. This demonstrated that a double bond, and not a ring structure, was present in the nonhydrogenated compound. GC-MS data from the pyrrolidide derivative of the unknown compound (Fig. 1) showed an interval of 12 amu between *m/z* 126 and *m/z* 138, indicating that the double bond was at the fourth carbon (9). The interval of 28 amu between the C-11 and C-12 fragments (*m/z* 236 and *m/z* 264) confirmed the location of the branched methyl group at carbon 12.

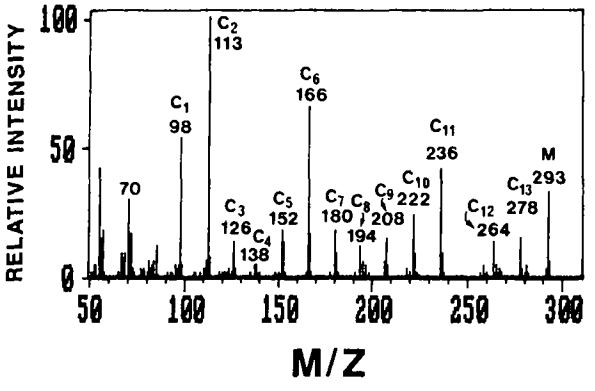

FIG. 1. Mass spectrum of the pyrrolidide derivative of 12-methyl-*cis*-4-tetradecenoic acid, extracted from *Corynebacterium sepedonicum*.

The infrared spectrum of the compound showed absorptions characteristic of unsaturated fatty acids: 3010 cm^{-1} (unsaturation), 2920 and 2860 cm^{-1} (C-H stretch), and 1745 cm^{-1} (ester carbonyl). No infrared absorption was observed at 965 cm^{-1}, due to *trans* double bonds. Therefore, the double bond at carbon 4 was determined to have the *cis* configuration, and the fatty acid was identified as 12-methyl-*cis*-4-tetradecenoic acid, a-15:1(4).

Bousfield et al. (12) alluded to the presence of a-15:1 in some bacteria, but did not report on the position of unsaturation. The characterization of this anteiso fatty acid is of interest because monounsaturation at carbon 4 is unusual among fatty acids from any source. This fatty acid is most likely the product of a Δ4-desaturase. Other possible biosynthetic routes include chain elongation or β-oxidation of other monounsaturated precursors. However, we could not detect the presence of the most probable monounsaturated precursors, 10-methyl-*cis*-2-dodecenoic acid or 14-methyl-*cis*-6-hexadecenoic acid, which might be converted to a-15:1(4) by elongation or β-oxidation, respectively.

The esterification reaction used in these experiments was conducted on whole extracts of saponified *C. sepedonicum* and not on isolated, individual lipid classes. Therefore, no conclusions can yet be drawn regarding the distribution of the various fatty acids among phospholipids, acylglycerols or free fatty acids. The extraction and esterification procedures used in these studies are widely used by clinicians and researchers for the rapid identification of bacteria and for determining their taxonomic relationships (1-4,7,8,12).

On the average, the a-15:1(4) content of *C. sepedonicum* was 5.9% of the total fatty acids (Table 1). We recently compared the fatty acid compositions of several *Corynebacteria* species and showed that the level of a-15:1(4) was significantly higher in *C. sepedonicum* than in the other species (4). Thus, the level of a-15:1(4) could serve as a useful marker to differentiate *C. sepedonicum* from other bacterial species.

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REFERENCES

1. Moss, C.W. (1981) *J. Chromatogr. 203,* 337-347.
2. Dees, S.B., Powell, J., Moss, C.W., Hollis, D.G., and Weaver, R.E. (1981) *J. Clin. Microbiol. 14,* 612-616.
3. De Boer, S.H. and Sasser, M. (1986) *Can. J. Microbiol. 32,* 796-800.
4. Gudmestad, N.C., Henningson, P.J., and Bugbee, W.M. (1988) *Can. J. Microbiol.,* in press.
5. Bugbee, W.M., Gudmestad, N.C., Secor, G.A., and Nolte, P. (1987) *Phytopathology 77,* 765-770.
6. De Boer, S.H., and Weiczorek, A. (1984) *Phytopathology 74,* 1431-1434.
7. Suzuki, I.K., and Komagata, K. (1983) *Int. J. Syst. Bacteriol. 33,* 188-200.
8. Miller, L., and Berger, T. (1985) Hewlett-Packard Gas Chromatography Application Note No. 228-41.
9. Andersson, B.A., and Holman, R.T. (1974) *Lipids 9,* 185-190.
10. Kaneda, T. (1977) *Bacteriol. Rev. 41,* 391-418.
11. Durham, D.R. (1978) *Int. J. Syst. Bacteriol. 28,* 223-228.
12. Bousfield, I.J., Smith, G.L., Dando, T.R., and Hobbs, G. (1983) *J. Gen. Microbiol. 129,* 375-394.

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Esterification of 8,11,14-Eicosatrienoate and Arachidonate Into Alkylacyl- and Diacylglycerophosphocholine by Vascular Endothelial Cells

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Agonist-stimulated phospholipases release arachidonate, but not 8,11,14-eicosatrienoate, from human endothelial cells. One source of the arachidonic acid is deacylation of 1-alkyl-2-arachidonoyl-glycerophosphocholine, with subsequent conversion of some of the resultant lysophospholipid to platelet-activating factor. This study has compared the distribution of incorporated 8,11,14-[^{14}C]-eicosatrienoate in alkylacyl-GPC and diacyl-GPC with that of [^{14}C]arachidonate synthesized endogenously by desaturation of the 8,11,14-[^{14}C]eicosatrienoate. Cells were incubated for 24 or 48 hr with 8,11,14-[^{14}C]eicosatrienoate, and the resultant mixture of ^{14}C-fatty acids in the cellular lipids was characterized by gas chromatography. The choline phospholipids were then separated, hydrolyzed with phospholipase C and derivatized to diradylbenzoates. Gas chromatographic analysis indicated extensive incorporation of [^{14}C]eicosatrienoate, as well as [^{14}C]arachidonate, into alkylacyl-GPC. Although the ratio of esterified [^{14}C]arachidonate to [^{14}C]eicosatrienoate was greater in alkylacyl-GPC than in diacyl-GPC, the enrichment with [^{14}C]arachidonate was far less than the ratio of arachidonate/eicosatrienoate released from these cells. These results thus support the hypothesis that the acyl specificity of polyunsaturated fatty acid release is provided by the agonist-stimulated phospholipase A_2 rather than the composition of the alkylacyl-GPC.
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Vascular endothelial cells are among the many types of cells that can synthesize 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine or platelet-activating factor (PAF), a potent hypotensive agent, as well as an active stimulant, of aggregation and degranulation of both platelets and neutrophils (1,2). The agonist-stimulated synthesis of PAF involves phospholipase A_2 activity on 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC), followed by the action of acetyltransferase on the lyso-PAF (1–3). In vascular endothelial cells, synthesis of PAF is initiated by agents such as histamine, bradykinin, thrombin and the calcium ionophore A23187 that also initiates mobilization of free arachidonic acid and synthesis of prostacyclin (4). These processes thus appear similar to those in human polymorphonuclear leukocytes, where 1-*O*-alkyl-2-arachidonoyl-GPC serves as a common source of PAF and arachidonate (5,6).

Our laboratory has recently demonstrated that the agonist-stimulated mobilization of arachidonic acid from endothelial phospholipids exhibits a high degree of acyl-group structural specificity (7–9). Polyunsaturated fatty acids which, like arachidonate, have a double bond in the delta-5 position are released in response to thrombin, histamine and the calcium ionophore A23187; 8,11,14-eicosatrienoate (20:3n-6), docosatetraenoate (22:4n-6) and other C_{20} and C_{22} polyunsaturated fatty acids without a delta-5 double bond are not released. Our working hypothesis has been that the specificity of agonist-stimulated fatty acid release is provided by the substrate specificity of the activated phospholipase A_2.

To further elucidate the mechanisms of arachidonate mobilization in vascular endothelial cells, we have investigated the incorporation of arachidonate and 8,11,14-eicosatrienoate in both diacyl- and ether-linked species of choline phospholipids. We postulated that 8,11,14-eicosatrienoate, like arachidonate, would be extensively esterified into 1-alkyl-2-acyl-GPC. Because 8,11,14-eicosatrienoate is not released from membrane phospholipids by agonists that mobilize arachidonate, extensive synthesis of 1-alkyl-2-eicosatrienoyl-GPC would be consistent with the proposed role of the phospholipase, A_2, in providing the requisite acyl specificity for agonist-stimulated release of polyunsaturated fatty acids. By contrast, a high ratio of arachidonate/8,11,14-eicosatrienoate in 1-alkyl-2-acyl-GPC relative to diacyl-GPC would provide evidence for specificity in the loading of this particular phospholipid pool.

MATERIALS AND METHODS

Radiolabeling of endothelial cells. Endothelial cells were obtained from human umbilical veins (10) and cultured in gelatin-coated 25 cm^2 flasks in HEPES-buffered Medium 199 plus 10% fetal bovine serum, 30 μg/ml Endothelial Cell Growth Supplement and 90 μg/ml heparin, as described previously (7,8). The experiments used confluent monolayers of second passage cells. 8,11,14-[^{14}C]Eicosatrienoate (55 mCi/mmol) was obtained from New England Nuclear (Boston, MA) and stored in hexane under nitrogen at −20°C. For each experiment, an aliquot of the ^{14}C-fatty acid solution was evaporated to dryness under N_2 and redissolved in 95% ethanol; the ethanol solution was added directly to fetal bovine serum. The endothelial cells were radiolabeled by incubation for 24 or 48 hr in complete growth medium supplemented with 5.0 μM 8,11,14-[^{14}C]eicosatrienoate; final concentration of ethanol in the culture medium was less than 0.2% (7,8). The cells were then washed with saline and harvested by rapid trypsinization (7).

Lipid extraction and analysis. Cellular lipids were extracted with acetone/ethyl acetate (11) and the extracts from 10–15 flasks pooled for analysis. Phospholipids were separated by one-dimensional thin layer chromatography (TLC) on Silica Gel H plates that were developed in chloroform/methanol/acetic acid/water (50:30:8:4, v/v/v/v). Lipid spots were visualized with iodine and scraped for quantitation of radioactivity by liquid scintillation

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Abbreviations: GPC, glycerophosphocholine; PAF, platelet-activating factor; TLC, thin layer chromatography.

spectrometry. For preparative separations, only the standard lanes, spotted with a mixture of phospholipids, were visualized with small drops of tincture of iodine. The choline phospholipids were then scraped and extracted from the silica gel (12).

Choline phospholipids were converted to diradylglycerobenzoates (diacyl, alkylacyl and alkenylacyl), as described by Blank et al. (13). This procedure involves hydrolysis with phospholipase C (14) and derivatization of the diradylglycerols with benzoic anhydride and 4-dimethylaminopyridine in benzene. The diradylglycerobenzoates were then separated by TLC on Silica Gel H plates developed with benzene/hexane/diethyl ether (50:45:4, v/v/v) (13). Standards prepared by hydrolysis and derivatization of diacyl-, alkyl- and plasmalogen or alkenylacyl phospholipids were chromatographed in adjacent lanes and visualized with iodine. The areas containing the diacyl and alkylacyl fractions were scraped and extracted from the silica gel with chloroform/methanol/diethyl ether (1:1:1, v/v/v).

Gas chromatography. Cellular lipid extracts, choline phospholipids and diradylglycerobenzoates were transesterified using methanolic base (12). The resultant ^{14}C-fatty acid methyl esters were separated by gas liquid chromatography and on-line radioactivity detection, as described previously (8,12).

RESULTS

In these studies, the endothelial cells were incubated with 5.0 μM 8,11,14-[^{14}C]eicosatrienoate (20 nmol/4 ml) for 24 or 48 hr. The relatively long incubation times were selected to examine the results of metabolic labeling of phospholipid pools rather than rapid fatty acyl turnover. Total incorporation of ^{14}C-fatty acyl groups into cellular glycerolipids was 64.5% after 24 hr and 57.3% after 48 hr. Because vascular endothelial cells actively desaturate [^{14}C]-eicosatrienoate to [^{14}C]arachidonate (12), long-term incubation with [^{14}C]eicosatrienoate results in a mixture of cellular ^{14}C-acyl groups. Cells incubated for 24 and 48 hr with [^{14}C]eicosatrienoate contained 38.8 and 51.4%, respectively, of the incorporated ^{14}C-acyl groups as [^{14}C]arachidonate. There was also substantial elongation of the synthesized [^{14}C]arachidonate to [^{14}C]docosatetraenoate (Table 1).

TABLE 1

Percentage Distribution of Incorporated ^{14}C in Cellular Fatty Acyl Groups of Endothelial Cells Incubated With 8,11,14-[^{14}C]Eicosatrienoate

	Incubation time	
Fatty acid	24 hr	48 hr
16:0	1.2[a]	1.6
20:3n-6	44.6	29.3
20:4n-6	38.8	51.4
22:3n-6	2.9	1.9
22:4n-6	12.6	15.8

[a]Values are means of triplicate determinations in one of two similar experiments; standard errors were <5% for larger peaks and <10% for smaller ones.

The choline-phospholipid fractions contained 39.0 and 33.6%, respectively, of the total cellular ^{14}C-fatty acyl groups in the 24 and 48 hr samples. As shown in Table 2, ca. 85% of the ^{14}C-fatty acids esterified in choline phospholipids were in diacyl-GPC and 10–11% in alkylacyl-GPC. These values are quite similar to those reported by Blank et al. (15) after a 4 hr incubation with [^{3}H]arachidonate; there was somewhat more incorporation into the alkylacyl fraction and less into the alkenylacyl fraction in the present study.

Table 3 shows the distribution of ^{14}C-fatty acyl groups in the diacyl- and alkyacylglycerobenzoate fractions. Both the diacyl- and alkylacyl-GPC fractions contained a mixture of ^{14}C-fatty acids. After both 24 and 48 hr of incorporation, the alkylacyl-GPC fraction contained sizable amounts of [^{14}C]eicosatrienoate. Thus, human endothelial cells readily incorporate 8,11,14-eicosatrienoate into alkylacyl-GPC. These cells also esterified substantial amounts of the [^{14}C]arachidonate and [^{14}C]docosatetraenoate synthesized from the incorporated 8,11,14-[^{14}C]eicosatrienoate into alkylacyl-GPC.

The results shown in Table 3 indicate some modest enrichment of alkylacyl-GPC with arachidonate, relative to diacyl-GPC, as shown by the higher ratios of [^{14}C]arachidonate/[^{14}C]eicosatrienoate in alkylacyl-GPC. Comparable results were obtained from a replicated 24 hr

TABLE 2

Percentage Distribution of Incorporated ^{14}C-Fatty Acids in Diradylglycerobenzoates Prepared From Endothelial Choline Phospholipids

	Incubation time	
Lipid class	24 hr	48 hr
Diacyl	84.4[a]	86.9
Alkylacyl	11.3	9.8
Alkenylacyl	4.3	3.8

[a]Values for each incubation time are means of the percentage distribution as determined from three separate aliquots of each cellular lipid extract.

TABLE 3

Percentage Distribution of ^{14}C-Fatty Acyl Groups in Diradylglycerobenzoates Prepared From Diacyl- and Alkylacylglycerophosphocholines of Endothelial Cells Incubated With 8,11,14-[^{14}C]Eicosatrienoate

	Incubation time			
	24 hr		48 hr	
Fatty acid	Diacyl	Alkylacyl	Diacyl	Alkylacyl
16:0	9.1[a]	4.7	14.3	4.3
20:3	61.4	49.2	29.8	26.7
20:4	26.3	36.2	44.5	59.0
22:3	0.8	4.6	2.5	0.8
22:4	3.2	5.3	8.9	9.5
Ratio 20:4/20:3	0.43	0.73	1.49	2.21

[a]Values are means from three independent derivatizations and analyses.

incubation, with ratios of 0.59 and 0.43 for the alkylacyl and diacyl fractions, respectively. In the replicated 48 hr experiment, the ratios were 1.24 and 0.85, respectively, for the alkylacyl and diacyl fractions; these values are lower than those in Table 3 due to less extensive desaturation of the incorporated [^{14}C]eicosatrienoate. Statistical analysis of the combined data (n = 4) using the Student's t-test for paired samples indicated that the ratio of [^{14}C]20:4/[^{14}C]20:3 in alkylacyl-GPC was significantly greater ($p < 0.05$) than that in diacyl-GPC.

DISCUSSION

This study has demonstrated extensive incorporation of 8,11,14-[^{14}C]eicosatrienoate, as well as [^{14}C]arachidonate, into alkylacyl-GPC of vascular endothelial cells. Although 8,11,14-eicosatrienoate is normally a minor component of endothelial phospholipids, the labeling procedure permits direct comparison of the patterns of esterification of 8,11,14-[^{14}C]eicosatrienoate with those of [^{14}C]arachidonate, synthesized endogenously by desaturation of the [^{14}C]eicosatrienoate. The results indicate quite similar patterns of utilization of 8,11,14-eicosatrienoate and arachidonate. Examination of [^{14}C]20:4/[^{14}C]20:3 ratios in alkylacyl-GPC and diacyl-GPC indicated a small but significant enrichment of alkylacyl-GPC with [^{14}C]arachidonate. Although substantially more [^{14}C]eicosatrienoate had been desaturated to [^{14}C]arachidonate by 48 hr than 24 hr, the extent of enrichment of alkylacyl-GPC with arachidonate was similar at 24 and 48 hr. This finding suggests that remodeling of the alkylacyl-GPC through deacylation-reacylation does not result in substantial replacement of 8,11,14-eicosatrienoate with arachidonate.

The modest enrichment of endothelial alkylacyl-GPC with arachidonate relative to other polyunsaturated fatty acids observed in the present study is consistent with findings in other cells. Compositional data from many cell types including neutrophils (16), macrophages (17,18) and mastocytoma cells (19) indicate that the alkylacyl-GPC fraction, although enriched with arachidonate, contains a mixture of *sn*-2 acyl groups. Indeed, loss of arachidonate from the *sn*-2 position of stimulated neutrophils is accompanied by rapid reacylation of some of the resultant lysophospholipid with linoleate and oleate (6). In contrast, inactivation of exogenous PAF through the sequential activities of acetylhydrolase and transacylase and incorporation of exogenous lyso-PAF is highly specific for reacylation with arachidonate in neutrophils (20), platelets (21–23) and macrophages (24). Recent results by Blank et al. indicate that endothelial cells may be somewhat limited in their ability to reacylate lyso-PAF and may thus synthesize alkylacyl-GPC primarily by the de novo route (25).

Our previous studies have demonstrated that the release of [^{14}C]arachidonate from endothelial cells stimulated with thrombin, histamine or the calcium ionophore A23187 is 8- to 12-fold more extensive than that of 8,11,14-[^{14}C]eicosatrienoate (7,8). The present study provides data which indicate extensive incorporation of 8,11,14-[^{14}C]eicosatrienoate into alkylacyl-GPC. There is some selectivity for esterification of [^{14}C]arachidonate, as opposed to 8,11,14-[^{14}C]eicosatrienoate into alkylacyl-GPC, as evidenced by higher ratios of [^{14}C]20:4/[^{14}C]20:3 in alkylacyl-GPC than diacyl-GPC at both 24 and 48 hr. The extent of enrichment of alkylacyl-GPC with [^{14}C]arachidonate relative to [^{14}C]eicosatrienoate is, however, not sufficient to provide the 10-fold acyl specificity observed in the agonist-stimulated release of arachidonate relative to eicosatrienoate (8,9). These results are thus consistent with the hypothesis that the fatty acyl specificity of arachidonate mobilization in endothelial cells is provided by the agonist-stimulated phospholipase A_2 activity. It is likely that the agonist-stimulated deacylation of endothelial phospholipids, like that in macrophages (5) and neutrophils (26), involves mobilization of arachidonate from both alkylacyl- and diacyl-GPC. Furthermore, stimulation of human umbilical-vein endothelial cells results in net loss of arachidonate from inositol-, as well as choline-phospholipids (27). There may also be contributions from ethanolamine-phospholipids and even triacylglycerols, as well (28). Indeed, recent evidence suggests that porcine endothelial cells (29), like neutrophils (30), mobilize substantial amounts of arachidonate from alkenylacyl-GPE, as well as from choline phospholipids.

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REFERENCES

1. Snyder, F. (1985) *Medicinal Res. Rev. 5*, 107–140.
2. Hanahan, D.J. (1986) *Ann. Rev. Biochem. 55*, 483–509.
3. Wykle, R.L., Malone, B., and Snyder, F. (1980) *J. Biol. Chem. 255*, 10256–10260.
4. McIntyre, T.M., Zimmerman, G.A., Satoh, K., and Prescott, S.M. (1985) *J. Clin. Invest. 76*, 271–280.
5. Albert, D.H., and Snyder, F. (1984) *Biochim. Biophys. Acta 796*, 92–101.
6. Chilton, F.H., Ellis, J.M., Olson, S.C., and Wykle, R.L. (1984) *J. Biol. Chem. 259*, 12014–12019.
7. Rosenthal, M.D., and Hill, J.R. (1986) *Biochim. Biophys. Acta 875*, 382–399.
8. Rosenthal, M.D. (1987) *Biochim. Biophys. Acta 917*, 279–289.
9. Rosenthal, M.D. (1988) *J. Cell. Physiol. 136*, 333–340.
10. Jaffe, E.A. (1980) *Transplant. Proc. 12*, Suppl. 1, 49–53.
11. Slayback, J.R.B., Cheung, L.W.Y., and Geyer, R.P. (1977) *Anal. Biochem. 83*, 372–384.
12. Rosenthal, M.D., and Whitehurst, M.C. (1982) *J. Cell. Physiol. 113*, 298–306.
13. Blank, M.L., Robinson, M., Fitzgerald, V., and Snyder, F. (1984) *J. Chromatogr. 298*, 473–482.
14. Mavis, R.D., Bell, R.M., and Vagelos, P.R. (1972) *J. Biol. Chem. 247*, 2835–2841.
15. Blank, M.L., Spector, A.A., Kaduce, T.L., and Snyder, F. (1986) *Biochim. Biophys. Acta 877*, 211–215.
16. Mueller, H.W., O'Flaherty, J.T., Greene, D.G., Samuel, M.P., and Wykle, R.L. (1984) *J. Lipid Res. 25*, 383–388.
17. Sugiura, T., Onuma, Y., Sekiguchi, N., and Waku, K. (1982) *Biochim. Biophys. Acta 712*, 515–522.
18. Ramesha, C.S., and Picket, W.C. (1987) *J. Lipid Res. 28*, 326–331.
19. Yoshioka, S., Nakashima, S., Okano, Y., Hasegawa, H., Ichiyama, A., and Nozawa, Y. (1985) *J. Lipid Res. 26*, 1134–1141.
20. Chilton, F.H., O'Flaherty, J.T., Ellis, J.M., Swendsen, C.L., and Wykle, R.L. (1983) *J. Biol. Chem. 258*, 7268–7271.
21. Malone, B., Lee, T.-C., and Snyder, F. (1985) *J. Biol. Chem. 260*, 1531–1534.
22. Kramer, R.M., Patton, G.M., Pritzker, C.R., and Deykin, D. (1983) *J. Biol. Chem. 258*, 13806–13811.
23. Homma, H., Kuman, R., and Hanahan, D.J. (1987) *Arch. Biochem. Biophys. 252*, 259–268.

24. Robinson, M., Blank, M.L., and Snyder, F. (1985) *J. Biol. Chem. 260,* 7889-7895.
25. Blank, M.L., Spector, A.A., Kaduce, T.L., Lee, T.-C., and Snyder, F. (1986) *Biochim. Biophys. Acta 876,* 373-378.
26. Swendsen, C.L., Ellis, J.M., Chilton, F.H., O'Flaherty, J.T., and Wykle, R.L. (1983) *Biochem. Biophys. Res. Commun. 113,* 72-79.
27. Hong, S.L., McLaughlin, N.J., Tzeng, C.-y., and Patton, G. (1985) *Thrombosis Res. 38,* 1-10.
28. Ragab-Thomas, J.M.-F., Hullin, F., Chap., H., and Douste-Blazy, L. (1987) *Biochim. Biophys. Acta 917,* 388-397.
29. Brown, M.L., Jakubowski, J.A., Leventis, L.L., and Deykin, D. (1987) *Biochim. Biophys. Acta 921,* 159-166.
30. Chilton, F.H., and Connell, T.R. (1988) *J. Biol. Chem. 263,* 5260-5265.

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Lipids

Notices
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23rd Annual Hugh Lofland Conference on Arterial Wall Metabolism (May 24–27, 1989, Welches, Oregon). Requests for information should be sent to M. R. Malinow, M.D., Chairman, Hugh Lofland Conference, Oregon Regional Primate Research Center, 505 NW 185th Avenue, Beaverton, OR 97006, (503) 690-5258.

10th Anniversary Meeting of the European Association for Cancer Research (University College, Galway, Ireland, September 11–13, 1989). For further information, contact Dr. S. M. Lavelle, Experimental Medicine, University College, Galway, Ireland; or in North America, contact Dr. J. H. Weisburger, American Health Foundation, Valhalla, New York, NY 10595-1599. The program involves plenary lectures, workshops, symposia and poster sessions.

REVIEW

Recent Advances in the Purification, Characterization and Structure Determination of Lipases

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Recently, lipases have been purified from mammalian, bacterial, fungal and plant sources by different methodologies. Purified lipases subsequently have been characterized for molecular size, metal binding capabilities, glycoside and phosphorus contents, and substrate specificities. Primary structures of several lipases have been determined either from amino acid or nucleic acid sequences. Lipases sequenced to date share sequence homologies including a significant region, Gly-X-Ser-X-Gly, that is conserved in all. The Ser residue is suspected to be essential for binding to lipid substrates.
Lipids 23, **1101-1106 (1988).**

Lipases are enzymes that catalyze the hydrolysis of triacylglycerols of animal fats and vegetable oils. The rapid development of lipase structure determination and application is coincident with a renewed interest in developing new domestic markets for U.S. surplus fats and oils. Recent reviews dealing with the potential for using lipases and other enzymes for industrial purposes abound (1-8). Because of this interest, a review of lipase purifications, characterizations and structure determinations to update the existing literature (9-11) seemed valuable.

ISOLATION AND PURIFICATION OF LIPASES

Mammalian lipases. Mammalian lipases from rat liver (12-14) and porcine and human pancreas (15-18) were recently investigated. Rat hepatic lipase was purified to homogeneity by two independent groups (12,13). The purification involved a multistep procedure including chromatography with heparin-Sepharose affinity chromatography, DEAE-Sephacel anion exchange chromatography, and gel filtration on Ultrogel AcA 34 by the former group (12), and octyl-Sepharose 4B, heparin-Sepharose 4B, hydroxyapatite and gel filtration by the latter (13). Twu et al. (13) also reported a purification using an immunoadsorbent column that had been prepared with rabbit anti-lipase antibodies coupled to Sepharose 4B. A yield of 10% lipase activity was obtained with any of the methods of purification. The native enzyme had M_r of 200,000, although when it was denatured it had M_r of 53,000, suggesting that the active enzyme is composed of four subunits. The native protein was shown to be a single enzyme molecule of broad substrate specificity toward triacylglycerols, monoacylglycerols and phosphoglycerols. The enzyme was devoid of free sulfhydryl groups at the active site because the use of *p*-chloromercuribenzoate, an alkylating agent for the -SH group, did not change the enzyme activity as assayed using triolein, monoolein and dipalmitoylphosphatidylcholine. However, diisopropylfluorophosphate, a serine inhibitor, did lead to loss of hydrolytic activity with all three substrates. Evidently serine residues are involved in the catalytic process.

Bacterial lipases. A lipase of *Staphylococcus aureus* was isolated and purified by a sequence involving ammonium sulfate precipitation, hydrophobic interaction chromatography with Phenylsepharose CL-4B, and gel filtration through Sepharose CL-4B (19). The enzyme had a M_r of 300,000 in the native state and 45,000 when it was denatured, suggesting subunit association in the native state. Amino acid analysis by acid hydrolysis indicated the presence of 31% hydrophobic residues (leucine, isoleucine, phenylalanine and valine), not very different from other proteins, but the absence of cysteine and methionine. Analysis of the nonprotein content indicated the presence of about 2% glycosidic residues, 0.05% of phosphorous and traces of lipids. The presence of phosphorous may be due to phospholipids bound to the hydrophobic amino acid residues of the lipase. The lipase showed both esterase activity with Tween 20 (a water soluble ester) and lipase activity with olive oil. The lipase preparation was stable within a pH range of 5.0 to 9.0 and exhibited maximal activity at pH 8.0. Chelating agents and metals such as Ca^{2+}, Cd^{2+} and Mn^{2+} had little effect on the activity of this enzyme, implying that the lipase is not a metalloenzyme.

A lipase from this same bacterial species, but from a different strain, has been sequenced very recently (20). From this sequencing study, Lee and Iandolo found that the molecular weight of their denatured enzyme was about 70,000 both by deduction from the DNA sequence and by SDS-polyacrylamide gel electrophoresis. In addition to the molecular weight difference between the two lipases, there were major differences in amino acid compositions that showed two quite different lipases were isolated from the same species of bacteria.

A lipase from *Pseudomonas aeruginosa,* a pathogenic bacterium, was purified by electrophoresis (21). This research group had been interested in characterizing that enzyme to determine whether the lipase made any contribution to the pathogenic properties of the bacteria. The lipase is extracellular and was present as high molecular weight aggregates (M_r = 500,000) that may be caused by association of lipopolysaccharides with the lipase. The lipopolysaccharides were, in fact, separated from the lipase by solubilization of the concentrated culture supernatant with the zwitterionic detergent CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. The solubilized enzyme was then separated on an isoelectric focusing gel, and a lipase band was subsequently eluted from the gel. The purified enzyme had a molecular weight of 29,000 and a pI value of 5.8. Lipase activity was measured photometrically with *p*-nitrophenylpalmitate or titrimetrically with olive oil as substrate. The

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HPLC, high performance liquid chromatography.

lipolytic activity appeared to depend on the presence of either the lipopolysaccharides or nonionic or zwitterionic detergents in the medium. In addition, lipase activity was stimulated by the presence of Ca^{2+} ions. Characterization of the properties of this lipase is in progress to determine possible contributons to the bacterial pathogenesis.

Fungal lipases. Two lipase isozymes (I and II) were isolated and purified from *Aspergillus niger* (22) by a combination of DEAE-Trisacryl M ion exchange chromatography, Sephadex G-50 gel filtration and hydrophobic chromatography using Phenylsepharose CL-4B. The motivation for these studies originated from the industrial use of the original lipase preparation, namely as a flavor developer in milk by-products. Technological lipase applications in the food industry can be more accurately guided once enzymatic properties are clearly established. The results of these characterizations are shown in Table 1.

The isozymes had somewhat different substrate specificities. Lipase I gave high rates of hydrolysis with nitrophenyl esters of C6-C12 fatty acids, while lipase II hydrolyzed esters of shorter chain fatty acids. The presence of $CaCl_2$ and $MgCl_2$ increased the activity of lipase I, but not of lipase II. Lipase I was inhibited by Ag^+ and iodoacetamide, while lipase II was inactivated by Ag^+ and Hg^{2+}. It is well established that metal binding sites can play a key role in the activity of an enzyme by influencing the conformation of the active site. The significance of these metals on the activation (inhibition) of these isozymes will be a matter for study. Inhibition of activity of Lipase I by reaction with iodoacetamide suggested the presence of a free sulfhydryl group at its active site. In addition to the differences already enumerated, the authors have also detected differences in the results from using lipases I and II in the development of compositions related to flavor.

A great deal of information was gathered on this enzyme. Unfortunately, the validity of the substrate and metal binding data are questionable. The lipase catalyzed hydrolysis of nitrophenyl esters of fatty acids is a two-step reaction. Initially a fatty acyl-lipase intermediate is formed, which then undergoes hydrolysis at a rate that is too fast to be measured by ordinary techniques (23).

Additionally, Tahoun et al. (24) report the isolation of an intracellular lipase of M_r 45,000, also from *A. niger.* Characterization of this protein was limited, but preliminary study revealed that the catalyst selected for short chain and unsaturated fatty acids and was able to esterify glycerol at both primary and secondary positions. On the other hand, the enzyme preferentially hydrolyzed acid residues from the primary glycerol position.

In pioneering work, Iwai and Tsujisaki (25) isolated and characterized three lipolytically active components from the fungus, *Rhizopus delemar.* In our own research, we have developed a preparative high performance liquid chromatography (HPLC) method (gel filtration) to affect a partial purification of crude *R. delemar* lipase (Miles Laboratories, Inc., Elkhart, IN). We observed 10 microheterogeneous proteins using two dimensional SDS-polyacrylamide gel electrophoresis of M_r = 38,900 with pI's ranging from 4.6 to 7.0. Amino acid analysis of this material revealed that the composition found for the *R. delemar* lipase is similar to that reported for *Rhizopus arrhizus* (26), except for the amount of Glx, which is higher in the former. Similarities observed among lipases from various sources can be of great value in understanding the catalytic mechanism of action of crucial amino acids, and the evolutionary relatedness of lipases. However, the existence of several differentially charged proteins casts doubt on previous reports of lipase purifications from *Rhizopus* species. More detailed structural studies on *R. delemar* lipase are in progress at the U.S. Department of Agriculture in Philadelphia, PA.

A lipase preparation from the fungus, *Mucor miehei* (recently classified as *Rhizomucor miehei* [27]) has received attention as a potentially useful catalyst for 1,3-positional interesterification (28,29). Recently, Huge-Jensen et al. (27,30) isolated two lipolytically active glycoproteins from this fungus that differed in degree of glycosylation. The steps in purification included chromatography of a crude precipitated powder on an anion-exchange column followed by either affinity chromatography (Con A-Sepharose) or hydrophobic interaction chromatography (Phenyl-Sepharose). Two lipase forms were obtained by independent sub-routines in the initial purification by anion exchange. The more highly glycosylated protein was converted to the less glycosylated variant in purification steps performed at low pH. Amino acid composition of both forms showed a similar protein backbone (27). Although the lipase forms differed in other properties, they both exhibited strong selectivity for the primary glycerol position in hydrolysis, favored longer (>C-10) fatty acid chains, and lacked triacylglycerol stereospecificity. The DNA sequence of both forms was very recently elucidated (27); they had the same primary structure.

Other lipase sources. Germinating oilseeds have been investigated by Hassanien and Mukherjee (31) as sources for lipases. Seeds generally contain a considerable amount of triacylglycerols that are processed by these lipases. Crude lipase preparations were isolated from seedling of rape *(Brassica napus)* and mustard *(Sinapis alba)* and cotyledons of lupine *(Lupinus albus)*; each showed

TABLE 1

Properties of Two Isozymes from *Aspergillus niger*

	Lipase I	Lipase II
M_r	31,000	19,000
pI	4.0	3.5
pH of maximal activity	5-6	5-6
% Carbohydrate content	6	9
Relative quantities of glycosides		
Mannose	26	20
Galactose	7	0
Glucose	5	10
Fucose	3	14
Arabinose	3	2
Hexosamine	2	13
Substrate specificity	long chains of fatty acids	short chains of fatty acids
Activity effectors		
$CaCl_2$	activated	unchanged
$MgCl_2$	activated	unchanged
Ag^+	inhibited	killed
Hg^{2+}	unchanged	killed
Iodoacetamide	inhibited	unchanged

specificity for the *sn*-1,3 positions of triacylglycerols with maximum activity between pH 8 and 9. In this work, crude lipase preparations were obtained by acetone or buffer solution extractions from easily grown seedlings. Locating active and stable lipases from plant sources has spurred interest in possible applications of plant lipases in enzymatic processing of fats and oils.

LIPASES: MOLECULAR STRUCTURE—MOLECULAR BIOLOGY

Mammalian lipases. The structural work on lipases is limited, and it seemed useful to consider a few of the earlier efforts in order to place the most recent research in perspective. The complete primary structure of a lipase was first determined by De Caro et al. (15) from the porcine pancreas. Earlier, partial lipase sequence data had been published by the same group (16,17). Amino acid sequencing by automated or manual Edman degradation was done on the cyanogen bromide fragmented enzyme. The porcine lipase was composed of a single chain of 449 amino acids. The calculated molecular weight of the protein moiety was about 50,000, and glycosidic residues gave a total molecular weight of 52,000 as observed by sedimentation equilibrium analysis. The glycan moiety of the lipase was bound to Asn-166 (16,32). Partial digestion of the glycopeptide ^{165}Thr-Asn-Gly-Thr-Ile-Glu-Arg^{171} yielded a glycopeptide containing only the ^{166}Asn-Gly-Thr^{168} fragment. Based on glycoprotein linkages determined previously, the oligosaccharide may be bound either to Asn or Thr. The oligosaccharide was not hydrolyzed by 0.5 N NaOH, which suggested that the site of glycosidation was Asn 166. The modification of an unidentified histidine residue by diethyl pyrocarbonate, forming carbethoxyhistidine, inactivated the lipase though the protein was able to adsorb on siliconized glass beads intact. This suggested that this residue was situated at, or close to, the active site (32). A serine residue that is located at position 152 was derivatized by reaction with diethyl *p*-nitrophenyl phosphate. This amino acid may be involved in substrate binding (33,34). Ser-152 occurs in the amino acid sequence **Gly-His-Ser-Leu-Gly**, a region that is homologous with other lipases; the bold-faced amino acids are highly conserved (Table 2).

The primary structure of human hepatic lipase was reported very recently (35). The lipase gene was mapped to the long arm of human chromosome 15. The amino acid sequence derived from the cDNA predicted a protein of 476 residues with calculated molecular weight of 53,249; it was preceded by a signal peptide of 23 residues. The lipase showed conservation of two hydrophobic domains in common with mammalian hepatic, lipoprotein and pancreatic lipases; **Gly-Tyr-Ser-Leu-Gly** and Ser-$(X)_6$-Ser regions were putative lipid-binding regions (35).

Human lipoprotein lipase was sequenced from a cDNA clone coding for a mature protein of 448 amino acids with

TABLE 2

A Conserved Sequence Among Lipolytic Enzymes

	Substrate binding region	Potential glycosylation sites N-X-T (X≠P)	Potential glycosylation sites N-X-S
	140 150		
Rat hepatic lipase	K V H L I G Y S L G A	1	1
	138 148		
Human hepatic lipase	H V H L I G Y S L G A	3	1
	125 135		
Human lipoprotein lipase	N V H L L G Y S L G A	1	1
	125 135		
Mouse lipoprotein lipase	N V H L L G Y S L G A	1	1
	127 137		
Bovine lipoprotein lipase	N V H L L G Y S L GA	1	1
	147 157		
Canine pancreatic lipase	Q V Q L I G H S L G A	0	1
	145 155		
Porcine pancreatic lipase	N V H V I G H S L G S	1	0
	174 184		
Human lecithin cholesterol acyltransferase	P V F L I G H S L G C	3	1
	146 156		
Rat lingual lipase	K I H Y V G H S Q G T	1	4
	362 372		
Staphylococcus hyicus	P V H F I G H S M G G	4	2
	405 415		
Staphylococcus aureus	K V H L V G H S M G G	4	3
	76 86		
Pseudomonas fragi	R V N L I G H S Q G A	0	0
	137 147		
Rhizomucor miehei	K V A V T G H S L G G	0	0

a calculated molecular weight of 50,394 (36). Lipoprotein lipase regulates the metabolism of plasma cholesterol and triacylglycerols. This lipase was 28% homologous to porcine pancreatic lipase, 46% to rat hepatic lipase and almost completely homologous to bovine lipoprotein lipase (Table 2).

Human lecithin-cholesterol acyltransferase cDNA-derived protein sequence was determined to have extensive homologies to mammalian lipases (37). This enzyme catalyzes the transfer of acyl groups from the *sn*-2 position of lecithin to the 3-OH of cholesterol. Physiologically, absence or malfunction of this enzyme results in the accumulation of cholesterol in the tissues. The mature protein contained 416 amino acids with a signal sequence of 24 residues. The calculated molecular weight of the amino acid backbone was 47,090. The enzyme contained the putative lipid binding region Gly-His-Ser-Leu-Gly that was present in different enzymes of lipid metabolism (Table 2).

Human pancreatic lipase was found to be a single polypeptide chain with M_r of 48,000 and has been partially sequenced (18). Specifically, amino acid residues at the N-terminus were sequenced; 21 of the residues showed homology with the N-terminus of porcine pancreatic lipase; 4 residues were not identical and 4 residues remain unidentified. Human lipase was also glycosylated. Complete primary structure of the human pancreatic lipase remains to be elucidated so that comparisons of functionality or structural homologies may be made with lipases of other origins.

Bovine lipoprotein lipase was another lipase investigated for its structure and function relationship. Sequences of NH_2-terminal and tryptic peptides were analyzed, revealing close homology of bovine lipoprotein lipase to porcine pancreatic lipase (38). One year later the complete primary structure was reported from the cDNA clone for lipoprotein lipase (39). The primary structure consisted of 450 amino acids in the unglycosylated form having a predicted molecular weight of 50,548. The enzyme had two hydrophobic regions, Gly-Tyr-Ser-Leu-Gly and Ser-$(X)_6$-Ser, that were present in other mammalian lipoprotein, hepatic and pancreatic lipases as well (Table 2) (35).

The complete sequence of canine pancreatic lipase mRNA was revealed (40). The enzyme was translated as a preenzyme with 467 amino acids, of which 17 residues compose the signal peptide. The mature lipase was calculated to have a molecular weight of 49,686. It showed 75.6% homology with porcine pancreatic lipase. The potential substrate binding site found in the other lipases was conserved here as well (Table 2).

Docherty et al. (41) have cloned and sequenced a rat lingual lipase. A preparation from the tongue serous glands was purified by chromatography on a carboxymethyl Sepharose CL6B column followed by gel filtration on Sephadex G100. The molecular weight of the partially deglycosylated enzyme (Endoglycosidase H) was ca. 41,000, and that of the glycosylated material was ca. 52,000. The purified lipase was used to produce rabbit polyclonal antibodies for the preparation of an immunoaffinity column for large scale purification of the lipase. These antibodies were also used for screening libraries.

A cDNA library was constructed in plasmids from mRNA isolated from the serous glands of rat tongue. Polyclonal antibodies against the purified lipase were used to identify the clone carrying the rat lipase gene. Subsequently, partial sequence analysis was done on the purified lipase to elucidate the N-terminal region. This sequence pinpointed the location of the signal peptide and that of the mature protein. A hydrophobic signal sequence of 18 residues cleaved to yield the mature lipase consisting of 377 residues.

This lipase shares sequence homology with porcine pancreatic lipase over a short region including a Ser-153 that coincides with the porcine pancreatic lipase Ser-152, that is essential for binding to lipid substrates (Table 2). A second and shorter segment of homology with porcine pancreatic lipase included an Asn-166, a putative glycosylation site in porcine pancreatic lipase as discussed previously and predicted to be such a potential site in the rat lingual lipase.

Rat hepatic lipase was purified, and partial amino acid sequence was determined by gas-phase microsequence analysis of proteolytic fragments (14). The enzyme had a molecular weight of ca. 52,700 obtained from SDS-polyacrylamide gels. The complete cDNA-derived amino acid sequence was obtained later with the help of an oligonucleotide probe synthesized on the basis of a partial sequence (42). The protein backbone was predicted to have a 53,222 molecular weight and was composed of 472 amino acids. The lipase was preceded by a hydrophobic leader sequence of 22 amino acids. The native lipase had an 8% (w/w) carbohydrate content (43). One of the two hydrophobic regions in common with other enzymes of lipid metabolism is shown in Table 2. The second hydrophobic sequence found in mammalian hepatic, lipoprotein and pancreatic lipases, Ser-$(X)_6$-Ser, was also present here.

cDNA-derived amino acid sequence of mouse lipoprotein lipase was also reported (44). The mature protein contained 447 amino acids with a predicted molecular weight of 50,314. Lipoprotein lipase is conserved extensively among mouse, human and bovine species. One of the putative substrate binding regions in homology with other enzymes of lipid metabolism is shown in Table 2.

Bacterial lipases. The lipase gene from *Staphylococcus hyicus hyicus* has been cloned and its sequence determined by Gotz et al. (45). The gene was cloned in plasmids, expressed in *S. carnosus,* and subcloned in *E. coli.* The DNA sequence yielded a preprotein of 641 amino acids. The authors predicted the location of the signal sequence based on homology with signal sequences from various organisms. The signal sequence was an N-terminal hydrophilic segment that was followed by a hydrophobic sequence ending in Ala-X-Ala that, finally, was followed by the sequence for the mature protein. Interestingly, the *S. hyicus hyicus* lipase also contained the sequence Phe-Ile-Gly-His-Ser-Met-Gly-Gly; the bold-faced region is homologous with lipase sequences from porcine pancreas and rat tongue (Table 2).

The lipase gene expressed in recombinantly transformed *S. carnosus* yielded an extracellular lipase with M_r value of 86,000, as determined by SDS-polyacrylamide gel electrophoresis. However, lipase excreted from donor strain *S. hyicus hyicus* showed a M_r of only 50,000. The authors attributed this molecular weight difference to an identified extracellular proteolytic activity in *S. hyicus hyicus.* The molecular weight calculated from

the preprotein amino acid sequence, including the signal sequence, is 71,000. The authors assumed the M_r 86,000 form enzyme may have hydrophilic or lipophilic contaminants leading to a reduced mobility in SDS gels and a high relative molecular weight.

The lipase gene from *S. aureus* was cloned and sequenced (19) as mentioned above. Detection of lipase activity in the cloned gene was on an egg yolk medium. A positive lipase activity was observed as a halo surrounding the colony upon accumulation of hydrolyzed fatty acids. To localize the lipase gene, various plasmids containing deletions at either end of the sequenced DNA fragment were generated. Deletions of DNA fragments allowed pinpointing the location of the gene. The DNA sequence revealed the presence of a signal sequence with the classical characteristics of a hydrophilic region followed by hydrophobic amino acids and ending in Ala-X-Ala prior to the mature protein. The predicted mature protein had a calculated basic pI of 9.3 and a M_r of 70,000 as determined by SDS-polyacrylamide gel electrophoresis. Comparison of the nucleotide sequences of the *S. aureus* and *S. hyicus hyicus* lipases revealed 46% homology, a significant amount. The amino acid sequences conserved between the two enzymes included the hydrophobic amino acid-rich region (residue 310 to the C terminus) that appeared to be associated with enzyme activity as observed by inactivation of the enzyme upon insertion of a lysogenic phage near the carboxyl end.

Finally, the sequence Leu-Val-Gly-His-Ser-Met-Gly-Gly was found in *S. aureus* lipase, and it contained the homologous region (boldface) found in all the lipases sequenced to date (Table 2).

Kugimiya et al. (46) cloned the lipase gene from *Pseudomonas fragi* in a plasmid. The lipase is known to have 1,3 positional specificity. The tributyrine diffusion agar method was used for the detection of lipase activity. DNA sequencing yielded a preprotein of 135 amino acids with a predicted M_r of about 15,000. A signal sequence composed of mostly hydrophobic amino acids was identified. Comparison of the lipase amino acid sequence with those from other organisms sequenced to date again showed the presence of the short homologous region Gly-His-Ser-X-Gly that is shared by porcine pancreatic, rat lingual, *S. hyicus* and *S. aureus* lipases, as seen in Table 2. In addition, another segment of amino acid sequence composed of five residues, falling in the signal portion, is homologous to the lipase from rat tonge.

Fungal lipases. The lipase gene from *Rhizomucor miehei* was cloned and sequenced (27) as mentioned above. The mature lipase was composed of 269 amino acids having a molecular weight of 29,472. The lipase was synthesized as part of a 363 residues-long precursor from which 24 amino acids were the signal sequence and 70 residues were the propeptide. The native enzyme was modified to two forms taking up either 11% (w/w) or 4% (w/w) carbohydrates. This lipase also showed sequence conservation in the vicinity of one Ser residue, located in a stretch of hydrophobic amino acid residues (27) (Table 2).

Finally, all sequenced lipases except those from *P. fragi* and *R. miehei* contain potential glycosylation sites (Table 2), N-X-T or N-X-S, where X cannot be proline (35). Oligosaccharides may link either through N-acetyl-D-glucosamine to the amide nitrogen of an Asn or through N-acetyl-D-galactosamine to the hydroxyl group of Ser or Thr.

CONCLUSION

We have entered an era in which the availability of protein sequence information, emerging data for substrate binding sites and the relationship of catalytically active residue(s) to the binding site direct us toward protein engineering. Site selective transformations through molecular biology techniques may increase greatly our understanding of lipase mode of action, and could provide semi-synthetic lipases having improved stability and/or desired substrate specificities. The prognosis for the immediate future appears to be continued research into the nature of lipases as an industrially important class of enzymes. A base of information currently is being generated that will stimulate and encourage old industries into evaluating new applications.

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REFERENCES

1. Aunstrup, K. (1979) *Appl. Biochem. Bioeng. 2,* 27-68.
2. Werdelmann, B.W., and Schmid, R.D. (1982) *Fette, Seifen, Anstrichm. 84,* 436-443.
3. Neidleman, S.L., and Geigert, J. (1984) *J. Am. Oil Chem. Soc. 61,* 290-297.
4. Boyce, C.O.L. (1984) *J. Am. Oil Chem. Soc. 61,* 1750-1753.
5. Posorske, L.H. (1984) *J. Am. Oil Chem. Soc. 61,* 1758-1760.
6. Ishida, S. (1984) *Yushi 37,* 47-51.
7. Nielsen, T. (1984) *Fette, Seifen, Anstrichm. 87,* 15-19.
8. Smith, H.V. (1985) *J. Am. Oil Chem. Soc. 62,* 351-355.
9. Brockerhoff, H., and Jensen, R.G. (1974) *Lipolytic Enzymes,* Academic Press, New York, NY.
10. Borgström, B., and Brockman, H.L. (1984) *Lipases,* Elsevier, New York, NY.
11. MacRae, A.R. (1983) in *Microbial Enzymes and Biotechnology* (Fogarty, W.M., ed.) pp. 225-249, Applied Science Publishers, New York, NY.
12. Jensen, G.L., Daggy, B., and Bensadoun, A. (1982) *Biochim. Biophys. Acta 710,* 464-470.
13. Twu, J.S., Garfinkel, A.S., and Schotz, M.C. (1984) *Biochim. Biophys. Acta 792,* 330-337.
14. Ben-Zeev, O., Ben-Avram, C.M., Wong, H., Nikazy, J., Shively, J.E., and Schotz, M.C. (1987) *Biochim. Biophys. Acta 919,* 13-20.
15. De Caro, J., Boudouard, M., Bonicel, J., Guidoni, A., Desnuelle, P., and Rovery, M. (1981) *Biochim. Biophys. Acta 671,* 129-138.
16. Bianchetta, J.D., Bidaud, J., Guidoni, A.A., Bonicel, J.J., and Rovery, M. (1979) *Eur. J. Biochem. 97,* 395-405.
17. Guidoni, A., Bonicel, J., Bianchetta, J., and Rovery, M. (1979) *Biochimie 61,* 841-845.
18. De Caro, A., Bonicel, J., Pieroni, G., and Guy, O. (1981) *Biochimie 63,* 799-801.
19. Tyski, S., Hryniewicz, W., and Jeljaszewicz, J. (1983) *Biochim. Biophys. Acta 749,* 312-317.
20. Lee, C.Y., and Iandolo, J.J. (1986) *J. Bacteriol. 166,* 385-391.
21. Stuer, W., Jaeger, K.E., and Winkler, U.K. (1986) *J. Bacteriol. 168,* 1070-1074.
22. Hofelmann, M., Hartmann, J., Zink, A., and Schreier, P. (1985) *J. Food Sci. 50,* 1721-1731.
23. Jencks, W.P. (1969) in *Catalysis in Chemistry and Enzymology,* pp. 597-599, McGraw-Hill Book Co.
24. Tahoun, M.K., El-Kady, M., and Wahba, A. (1985) *Microbios. Lett. 28,* 133-139.
25. Iwai, M., and Tsujisaki, Y. (1974) *Agric. Biol. Chem. 38,* 1241-1247.

26. Benzonana, G. (1974) *Lipids 9,* 166-172.
27. Boel, E., Huge-Jensen, B., Christensen, M., Thim, L., and Fiil, N.P. (1988) *Lipids 23,* 701-706.42.
28. Hansen, T.T., and Eigtved, P. (1986) in *Proceedings of the World Conference on Emerging Technologies in the Fats and Oils Industries* (Baldwin, A.R., ed.) pp. 365-369, American Oil Chemists' Society, Champaign, IL.
29. Eigtved, P., Hansen, T.T., and Huge-Jensen, B. (1985) in *Proceedings of the 13th Scandinavian Symposium on Lipids,* Scandinavian Forum, *Lipids Res. Technol.,* pp. 135-148, Goteborg, Sweden.
30. Huge-Jensen, B., Galluzzo, D.R., and Jensen, R.G. (1987) *Lipids 22,* 559-565.
31. Hassanien, F.R., and Mukherjee, K.D. (1986) *J. Am. Oil Chem. Soc. 63,* 893-897.
32. Plummer, T.H., and Sarda, L. (1973) *J. Biol. Chem. 248,* 7865-7869.
33. Chapus, C., and Semeriva, M. (1976) *Biochemistry 15,* 4988-4991.
34. Guidoni, A., Benkouka, F., De Caro, J., and Rovery, M. (1981) *Biochim. Biophys. Acta 660,* 148-150.
35. Datta, S., Luo, C., Li, W., Van Tuinen, P., Ledbetter, D.H., Brown, M.A., Chen, S., Liu, S., and Chan, L. (1988) *J. Biol. Chem. 263,* 1107-1110.
36. Wion, K.L., Kirchgessner, T.G., Lusis, A.J., Schotz, M.C., and Lawn, R.M. (1987) *Science 235,* 1638-1641.
37. McLean, J., Fielding, C., Drayna, D., Dieplinger, H., Baer, B., Kohr, W., Henzel, W., and Lawn, R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83,* 2335-2339.
38. Ben-Avram, C.M., Ben-Zeev, O., Lee, T.D., Haaga, K., Shively, J.E., Goers, J., Pedersen, M.E., Reeve, J.R. Jr., and Schotz, M.C. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83,* 4185-4189.
39. Senda, M., Oka, K., Brown, W.V., Qasba, P., and Furuichi, Y. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84,* 4369-4373.
40. Kerfelec, B., LaForge, K.S., Puigserver, A., and Scheele, G. (1986) *Pancreas 1,* 430-437.
41. Docherty, A.J.P., Bodmer, M.W., Angal, S., Verger, R., Riviere, C., Lowe, P.A., Lyons, A., Emtage, J.S., and Harris, T.J.R. (1985) *Nucleic Acid Res. 13,* 1891-1903.
42. Komaromy, M.C., and Schotz, M.C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84,* 1526-1530.
43. Augustin, J., Freeze, H., Tejada, P., and Brown, W.V. (1978) *J. Biol. Chem. 253,* 2912-2920.
44. Kirchgessner, T.G., Svenson, K.L., Lusis, A.J., and Schotz, M.C. (1987) *J. Biol. Chem. 262,* 8463-8466.
45. Gotz, F., Popp, F., Korn, E., and Schleifer, K.H. (1985) *Nucleic Acid Res. 13,* 5895-5906.
46. Kugimiya, W., Otani, Y., Hashimoto, Y., and Takagi, Y. (1986) *Biochem. Biophys. Res. Commun. 141,* 185-190.

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ARTICLES

Effect of Host Sterols on the Sterol Composition and Virulence of a Nuclear Polyhedrosis Virus of *Heliothis zea*

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The type of sterol in the diet of *Heliothis zea* affected not only the sterol composition of the insect larva but also the virulence and/or sterol composition of a single-nucleocapsid nuclear polyhedrosis virus (HzSNPV). This baculovirus, which was purified by differential and sucrose density gradient centrifugation, had a sterol content of 40 ng per 10^6 polyhedra. When the sterol composition of HzSNPV was characterized by gas liquid chromatography, reversed phase-high performance liquid chromatography, mass spectrometry, proton nuclear magnetic resonance spectrometry and/or ultraviolet spectroscopy, the sterols in the virus were similar to those of the host. The HzSNPV isolated from larvae fed Δ^5-, Δ^0- or $\Delta^{5,7}$-sterols contained primarily cholesterol, cholestanol or 7-dehydrocholesterol, respectively. Changes in the sterol composition of HzSNPV affected its LD_{50}, but not LT_{50}, in larvae containing Δ^5-sterol. The LD_{50} of virus isolated from larvae containing Δ^0-, Δ^5- and Δ^7-sterols decreased from 275,423 to 32,359 to 5,012 polyhedra/larva, respectively. The latter virus was also more virulent than the one that was isolated from larvae containing $\Delta^{5,7}$-sterol and had an LD_{50} of 58,884 polyhedra/larva. In contrast, the LD_{50} of an HzSNPV (Sandoz, Inc.) containing Δ^5-sterols was not affected by the presence of Δ^5-, Δ^0- or $\Delta^{5,7}$-sterols in the tissues of the host (1,413; 1,288 and 355 polyhedra/larva, respectively). The results of this study indicate that the sterol composition of *H. zea* can affect the sterol composition of HzSNPV and therefore may affect the ability of this biological control agent to control its economically important insect host. *Lipids 23,* 1107-1113 (1988).

The functional role of sterols in viruses is still poorly understood. The sterols in the envelope of viruses probably function as important structural components, just as they do in the membranes of cells (1). Sterols may be necessary in the envelope to assure that the virions are released from the host cell and/or can enter other susceptible cells (2). Interestingly, the depletion of cholesterol from vesicular stomatitis virus caused a substantial loss of infectivity of the virions (3,4). Although the sterol composition of some viruses has been determined (5), the quantity and type of sterol in many other enveloped viruses (e.g., baculoviruses of insects) is unknown.

Viruses are not known to carry the genes responsible for the synthesis of enzymes involved in lipid synthesis (1). Therefore, the lipids such as sterols in the envelope appear to be derived from the host cell. This suggests that if the sterol composition of an organism changed, the sterol composition of the virus might be affected. Bates and Rothblat (6) found that when vesicular stomatitis virus was grown in L cells, which were different from normal cells grown in tissue culture because they contained desmosterol instead of cholesterol, the virus contained only desmosterol and no cholesterol. The stability, growth and plaquing efficiency of the virus, however, was not affected by the presence of this $\Delta^{5,24}$-sterol. In contrast, the fusion of certain viruses (e.g., Semliki Forest virus [SFV] [7], Sindbis virus [8] and Sendai virus [9]) to liposomes was dependent on the quantity or structure of the sterol present in the liposome. When androstanol, cholestanol, coprostanol or sterols lacking a 3β-OH group were used in place of cholesterol in the liposome, the percentage of fusion of SFV with the liposome was reduced to 88.5, 53, 40 and $\leq$13% of that of cholesterol, respectively (7). Therefore, further studies of the effect of host sterols on the sterol composition and infectivity of viruses are warranted.

Many animals contain cholesterol as their tissue sterol, because they can synthesize this molecule from acetate (10), and so their enveloped viruses also usually contain this sterol (11). However, some invertebrates, such as insects, cannot synthesize sterols de novo and so obtain them exogenously in order to synthesize membranes and hormones (e.g., ecdysteroids) (12). Thus, the sterol composition of an insect may vary depending on its ability to utilize and/or metabolize different exogenous sterols (e.g., phytosterols) in its tissues. For example, the principal tissue sterols in the larva of the corn earworm, *Heliothis zea,* are cholestanol, cholesterol, 7-dehydrocholesterol or lathosterol when various Δ^0-, Δ^5-, $\Delta^{5,7}$- or Δ^7-24-alkyl- and desalkylsterols are its dietary sterols, respectively (13,14). Therefore, it is of interest to know whether changes in the sterol composition of insects can affect the sterol composition and/or virulence of their enveloped viruses, particularly those that are biological control agents of insect pests or are vectored by them.

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Abbreviations: a_c, k' for the test sterol/k' for cholesterol; TMS, $Si(CH_3)_4$; χ^2, chi-square; df, degrees of freedom; EM, electron microscopy; GLC, gas liquid chromatography; ^{1}H-NMR, proton nuclear magnetic resonance spectrometry; HzSNPV, single-nucleocapsid nuclear polyhedrosis virus of *H. zea*; k', $(V_t - V_0)V_0$; LD_{50}, median lethal dose; LT_{50}, median lethal time; MS, mass spectrometry; NPV, nuclear polyhedrosis virus; RP-HPLC, reversed phase-high performance liquid chromatography; RRT, retention time relative to cholesterol; TLC, thin layer chromatography; UV, ultraviolet spectroscopy; V_0, void volume; V_t, retention volume of the sterol.

The systematic nomenclature for the sterols referred to by their trivial names is: cholestanol, 5α-cholestan-3β-ol; cholesterol, cholest-5-en-3β-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; 22-dihydroergosterol, 24β-methylcholesta-5,7-dien-3β-ol; ergosterol, 24β-methylcholesta-5,7,22-trien-3β-ol; lathosterol, cholest-7-en-3β-ol; 24ξ-methylcholesterol, 24ξ-methylcholesta-5-en-3β-ol; sitosterol, 24α-ethylcholest-5-en-3β-ol; stigmasterol, 24α-ethylcholest-5,22-dien-3β-ol.

Cholesterol is the only sterol that has been reported from those enveloped insect viruses which have been studied (e.g., *Chilo* iridescent virus [15]). Although the nuclear polyhedrosis viruses (NPV) of *Bombyx mori* (16), *Lymantria dispar* (17) and *Pseudaletia unipuncta* (18) are known to contain lipids (e.g., phospholipids), the sterol composition of this type of virus has never been investigated.

Consequently, the purpose of the present study was: (a) to determine the quantity of sterol in a single-nucleocapsid nuclear polyhedrosis virus of *H. zea* (HzSNPV); (b) to investigate whether infection with HzSNPV alters the normal metabolism of sterols in *H. zea* larvae; (c) to determine how the sterol composition of the insect affects the sterol composition of the baculovirus; and (d) to study how the sterol composition of HzSNPV affects its virulence.

MATERIALS AND METHODS

H. zea. A disease-free colony of *H. zea* was established from eggs kindly provided by Dr. N. C. Leppla (USDA, Gainesville, FL) and maintained as described previously (19). The experimental larvae were fed a variety of artificial diets which affected their sterol composition. Some larvae were reared on medium that contained cholesterol (recrystallized twice from ethanol; 50 mg/100 ml diet; J. T. Baker Chemical Co., Phillipsburg, NJ) and was supplemented with wheat germ and Mazola corn oil (19); the principal tissue sterol in these larvae was cholesterol (20). Other larvae were fed diets which contained ergosterol (recrystallized twice from ethanol; ICN Pharmaceuticals, Inc., Cleveland, OH), cholestanol (Applied Science Laboratories, State College, PA) or lathosterol (Research Plus Steroid Laboratories, Bayonne, NJ) (20 mg/100 ml diet) and were supplemented with linoleic and linolenic acid (21); the principal tissue sterols in these larvae were 7-dehydrocholesterol (14), cholestanol or lathosterol, respectively (13).

Propagation, isolation and purification of HzSNPV. HzSNPV, a gift from Dr. T. R. Shieh (Sandoz, Inc., Homestead, FL), was propagated in larvae of *H. zea*, which contained a variety of tissue sterols. The 5th-instar larvae were inoculated per os (22) with 1.0×10^5 to 1.0×10^7 polyhedra/4 μl H_2O, using a blunt 30-gauge needle attached to a 1/4 ml syringe mounted on a microburet (Micro-Metric Instrument Co., Tampa, FL). Five days later, the moribund larvae were collected and frozen until the HzSNPV was isolated from the tissues and purified by differential and sucrose density centrifugation. The larvae were thawed, triturated in H_2O, the homogenate filtered through cheesecloth and the filtrate centrifuged at 11,872 *g*. The pellet was resuspended in H_2O and centrifuged at 475 *g* for 10 min. The supernatant containing the HzSNPV was then centrifuged at 11,872 *g* for 20 min to pellet the polyhedra. The virus was layered onto sucrose step gradients (consisting of equal amounts of 50 and 80% sucrose) and centrifuged at 54,000 *g* for 2 hr. The band containing the polyhedra was then collected and the polyhedra washed and lyophilized.

Electron microscopy of HzSNPV. Fat body and tracheae from infected larvae containing cholesterol were prepared for electron microscopy (EM) and examined using a Philips EM 300 electron microscope, as described previously (23), in order to confirm that the virus was a single-nucleocapsid NPV.

Quantitation of insect tissue sterol associated with HzSNPV. The amount of exogenous sterol from the host tissues, which was associated with the purified HzSNPV, was determined by combining an equal weight of infected larvae (fed unlabeled cholesterol) with noninfected larvae (fed radiolabeled cholesterol) and measuring the amount of radioactivity associated with the HzSNPV isolated from the mixture. The infected larvae were reared on diet that contained unlabeled cholesterol (15 mg/100 ml) and was supplemented with linoleic and linolenic acid (19). The noninfected larvae were reared on a similar diet that also contained [7-^{3}H]cholesterol (37 Ci/mmol; New England Nuclear, Boston, MA). The radiolabeled sterol was diluted with the unlabeled cholesterol so that the final specific activity of cholesterol in this diet was 2.58 mCi/mmol. The polyhedra were isolated from the tissues, as described above, and the number of polyhedra/ml calculated using a bacteria counter (C.A. Hausser and Son, Philadelphia, PA). The amount of exogenous sterol associated with the virus was determined by solubilizing the purified polyhedra (5.93 to 16.70×10^6) in 0.5 ml Protosol (New England Nuclear), neutralizing the solution with glacial acetic acid to prevent chemiluminescence, suspending it in 10 ml of 3a20 scintillation cocktail (Research Products International Corp., Mount Prospect, IL) and measuring the amount of radioactivity present with an LS 7500 liquid scintillation counter (Beckman Instruments, Irvine, CA). The counts were corrected for quench and background and the $\pm$ values represent the standard deviation of the mean.

Quantitation of sterol in insect tissue. In order to confirm that sterol was present in the insect tissues, samples of fat body (1.0 to 2.8 mg) were isolated from larvae fed the diet containing [7-^{3}H]cholesterol, weighed and digested in 1 ml of Protosol. The solution was then neutralized and the amount of radioactivity/mg of tissue determined by scintillation counting.

Quantitation of sterol in HzSNPV. The total amount of sterol associated with the polyhedra was determined by isolating the virus from infected larvae reared on diet containing [7-^{3}H]cholesterol and measuring the amount of radioactivity associated with the polyhedra (1.35 to 2.03×10^6), as previously described. The proportion of sterol that was probably exogenous was then subtracted from this value in order to determine the amount of endogenous sterol present in HzSNPV.

Bioassays. Fifth-instar larvae that contained various tissue sterols were inoculated per os with HzSNPV, which had been propagated in larvae containing various tissue sterols or obtained from Sandoz Inc., to ascertain the effect of the different sterols on the virulence (i.e., the LD_{50}) of the virus. The various doses of HzSNPV were prepared using the bacteria counter to determine the number of polyhedra/ml. The larvae were examined daily for the signs and symptoms of disease. In each bioassay, the median lethal time (LT_{50}) of the virus was determined from the time course of infection and the median lethal dose (LD_{50}) calculated using probit analysis (24).

Isolation of sterols from HzSNPV. The lipids from HzSNPV (Sandoz, Inc.) were obtained by continuous extraction of the polyhedra with acetone in a Soxhlet apparatus for 24 hr. The lipids from other HzSNPV (isolated

from larvae of *H. zea*) were obtained using a modified Bligh-Dyer technique (25). The lyophilized polyhedra were hydrated, suspended in chloroform-methanol, incubated at 60°C for 15 to 20 min and the filtrates from 3 extractions pooled. The lipids in both the acetone and chloroform-methanol extracts were saponified in 5% KOH in 90% ETOH during refluxing for 1 hr or at 60°C overnight. The neutral lipids were then extracted with diethyl ether. Due to the small sample size, the 4-desmethylsterols were rapidly isolated by preparative thin layer chromatography (TLC) on Silica Gel G using a solvent system of benzene and ethyl acetate (9:1, v/v). Some of the isolated 4-desmethylsterols from the Sandoz HzSNPV were then separated from one another via preparative reversed phase-high performance liquid chromatography (RP-HPLC) using a Perkin-Elmer Series 1 liquid chromatograph, equipped with a variable wavelength UV detector and a Perkin-Elmer preparative C_{18} column at 30°C. The mobile phase was 100% acetonitrile at a flow rate of 10 ml/min. The sample was injected in 2 ml of isopropanol and the sterols collected in 10 ml fractions.

Isolation of sterols from infected larvae. Larvae of *H. zea* were homogenized with a Sorvall Omni-Mixer (Dupont Instruments, Newtown, CT), the lipids extracted with chloroform-methanol and saponified, as previously described. The neutral lipids were extracted with diethyl ether and the 4-desmethylsterols isolated by TLC or column chromatography on silicic acid (10 mg lipid/g adsorbent; Unisil, Clarkson Chemical Co., Inc., Williamsport, PA). The latter procedure utilized an elution scheme of increasing amounts of ether in hexane and the eluent was monitored by TLC and gas liquid chromatography (GLC). Some or all of the isolated 4-desmethylsterols were then separated from one another via preparative RP-HPLC, as previously described.

Identification of sterols. The sterols isolated from HzSNPV and infected larvae were characterized by GLC, RP-HPLC, ultraviolet spectroscopy (UV), mass spectrometry (MS) and/or proton nuclear magnetic resonance spectrometry (^{1}H-NMR) and identified after comparing their characteristics to those of the dietary sterols and standards (e.g., cholestanol, cholesterol, 7-dehydrocholesterol [Sigma Chemical Co., St. Louis, MO], desmosterol [Sigma Chemical Co.], ergosterol, lathosterol, 24ξ-methylcholesterol [Research Plus Steroid Laboratories], sitosterol [Sigma Chemical Co.], and stigmasterol [Sigma Chemical Co.]) and/or the published descriptions of sterols (e.g., 22-dihydroergosterol [26], Δ^7-24ξ-methylcholestenol, and $\Delta^{7,22}$-24ξ-methylcholestenol [27]).

GLC was performed using a Perkin-Elmer Sigma 3B chromatograph and a 1% SE-30 and/or a 3% QF-1 column at 230 to 235°C. Rates of movement of the sterols were expressed as retention times relative to cholesterol (RRT) ± the actual range of the results. Analytical RP-HPLC was performed on a Perkin-Elmer Series 3B liquid chromatograph equipped with a variable wavelength UV detector, stopflow capabilities and a Zorbax ODS (C_{18}) column at 45°C. The mobile phase was acetonitrile/isopropanol (80:20, v/v) at a flow rate of 2 ml/min. The k′ value ($[V_t - V_0]/V_0$ where V_t is the retention volume of the sterol and V_0 is the void volume) for cholesterol was determined and used to calculate the α_c values (k′ for the test sterol/k′ for cholesterol) of the experimental peaks. The ± values associated with the α_c's represent the actual range of the results. Ultraviolet absorption spectra were obtained by scanning the individual peaks from analytical RP-HPLC between 200 and 300 nm. MS was performed via direct probe on a model 4000 Finnigan instrument with electron impact ionization at 70 eV. The ± values associated with the percentage of the ions present represent the actual range of the results. ^{1}H-NMR was performed at 360 MHz at ambient temperature on a Bruker instrument, model WH360; up to ca. 1 to 2 mg of sterol was dissolved in $CDCl_3$ and $Si(CH_3)_4$ (TMS) was the internal standard.

RESULTS

Identification of sterols. The characteristics of the sterols isolated in this study are summarized below:

Cholestanol. The RRTs in GLC were 1.09 ± 0.02 on QF-1 and 1.00 ± 0.02 on SE-30. The *m/e* of the ions in MS were: 388 (M^+, 22%), 373 (M^+-CH_3, 11%), 355 ($M^+-CH_3-H_2O$, 4%), 273 (M^+-SC-2H, 1%), 257 (M^+-SC-H_2O, 2%), 233 (M^+-SC-C_3H_6, 79%), 231 (M^+-SC-C_3H_8, 7%) and 215 (M^+-SC-$C_3H_6-H_2O$, 100%). The chemical shifts, in ppm from TMS, of protons in ^{1}H-NMR were: C-18, 0.64 (s); C-19, 0.80 (s); C-21, 0.90 (d, J = 7 Hz); C-26 and 27, 0.86 and 0.86 (d, J = 6 Hz).

Cholesterol. The RRTs in GLC were 1.00 ± 0.01 on QF-1 and SE-30. The α_c in RP-HPLC was 1.00 ± 0.01 with an UV absorption maximum at 208 nm. The *m/e* of the ions in MS and the chemical shifts, in ppm from TMS, of protons in ^{1}H-NMR were similar to published values (27).

7-Dehydrocholesterol. The RRTs in GLC were 1.12 ± 0.01 on QF-1 and 1.08 ± 0.01 on SE-30. The α_c in RP-HPLC was 0.77 ± 0.01 with UV absorption maxima at 208, 273, 283 and 295 nm. The *m/e* of the ions in MS and the chemical shifts, in ppm from TMS, of protons in ^{1}H-NMR were similar to published values (27).

Desmosterol. The α_c in RP-HPLC was 0.69 ± 0.01 with a UV absorption maximum at 208 nm. The *m/e* of the ions in MS were: 384 (M^+, 78%), 369 (M^+-CH_3, 44%), 273 (M^+-SC-2H, 100%) and 253 (M^+-SC-H_2O-2H, 11%).

22-Dihydroergosterol. The α_c in RP-HPLC was 0.86 ± 0.01 with UV absorption maxima at 208, 272, 283 and 294 nm. The *m/e* of the ions in MS were: 398 (M^+, 37 ± 21%), 383 (M^+-CH_3, 7%), 380 (M^+-H_2O, 6 ± 6%), 365 ($M^+-CH_3-H_2O$, 41 ± 33%), 339 ($M^+-C_3H_5-H_2O$, 23 ± 19%), 337 ($M^+-C_3H_7-H_2O$, 5 ± 1%), 271 (M^+-SC, 59 ± 11%), 253 (M^+-SC-H_2O, 37 ± 22%), 211 (M^+-SC-$C_3H_6-H_2O$, 52 ± 30%) and 209 (M^+-SC-$C_3H_8-H_2O$, 12 ± 5%). The chemical shifts, in ppm from TMS, of protons in ^{1}H-NMR were: C-18, 0.62 (s); C-19, 0.94 (s); C-21, 0.93 (d, J = 7 Hz); C-26 and 27, 0.79 and 0.78 (d, J = 7 Hz); C-28, 0.86 (d, J = 6 Hz).

Ergosterol. The RRTs in GLC were 1.21 ± 0.01 on QF-1 and 1.20 ± 0.01 on SE-30. The α_c in RP-HPLC was 0.72 ± 0.01 with UV absorption maxima at 208, 272, 283 and 294 nm. The *m/e* of the ions in MS and the chemical shifts, in ppm from TMS, of protons in ^{1}H-NMR were similar to published values (27).

24ξ-Methylcholesterol. The RRTs in GLC were 1.29 ± 0.01 on QF-1 and 1.30 ± 0.05 on SE-30. The α_c in RP-HPLC was 1.12 ± 0.02 with a UV absorption maximum at 208 nm. The *m/e* of the ions in MS were: 400 (M^+, 100%), 385 (M^+-CH_3, 26%), 382 (M^+-H_2O, 30%),

367 (M^+-CH_3-H_2O, 22%), 315 (M^+-85, 26%), 289 (M^+-C_7H_{11}, 26%), 273 (M^+-SC, 9%), 255 (M^+-SC-H_2O, 17%), 231 (M^+-SC-C_3H_6, 9%), 229 (M^+-SC-C_3H_8, 9%) and 213 (M^+-SC-C_3H_6-H_2O, 22%). The chemical shifts, in ppm from TMS, of protons in ^{1}H-NMR were: C-18, 0.68 (s); C-19, 1.00 (s); C-21, 0.90 (d, J = 7 Hz); C-26 and 27, 0.77 and 0.84 (d, J = 6 Hz); C-28, 0.79 (d, J = 7 Hz) for 24α-methylcholesterol and C-18, 0.68 (s); C-19, 1.00 (s); C-21, 0.91 (d, J = 7 Hz); C-26 and 27, 0.85 and 0.78 (d, J = 7 Hz); C-28, 0.79 (d, J = 7 Hz) for 24β-methylcholesterol.

Sitosterol. The RRTs in GLC were 1.53 ± 0.02 on QF-1 and 1.63 ± 0.02 on SE-30. The α_c in RP-HPLC was 1.25 ± 0.02 with a UV absorption maximum at 208 nm. The *m/e* of the ions in MS and the chemical shifts, in ppm from TMS, of protons in ^{1}H-NMR were similar to published values (27).

Stigmasterol. The RRTs in GLC were 1.29 ± 0.01 on QF-1 and 1.44 ± 0.09 on SE-30. The α_c in RP-HPLC was 1.08 ± 0.00 with a UV absorption maximum at 208 nm. The *m/e* of the ions in MS and the chemical shifts, in ppm from TMS, of protons in ^{1}H-NMR were similar to published values (27).

Sterols from infected larvae. The principal sterols present in infected insects in this study were similar to those found previously in noninfected larvae (13,14,20). The sterols isolated from 25.1 g of infected larvae-fed diet containing cholesterol (Table 1) were 95% cholesterol, <1% 24α- and 24β-methylcholesterol, 3% sitosterol, <1% stigmasterol (characterized by GLC, RP-HPLC, UV, MS and ^{1}H-NMR with the exception of stigmasterol which was characterized by MS and ^{1}H-NMR, only) and <1% desmosterol (characterized by RP-HPLC and MS).

The sterols isolated from 20.4 g of infected larvae-fed diet containing cholestanol (Table 1) were >95% cholestanol (characterized by GLC, MS and ^{1}H-NMR) and <5% cholesterol (characterized by RP-HPLC).

The sterols isolated from 56.7 g of infected larvae-fed diet containing ergosterol (Table 1) were 8 ± 4% cholesterol, 50 ± 8% 7-dehydrocholesterol, 37 ± 7% ergosterol (characterized by GLC, RP-HPLC, UV, MS and ^{1}H-NMR), 6 ± 3% lathosterol plus Δ^7-24ξ-methylcholestenol plus $\Delta^{7,22}$-24ξ-methylcholestenol, <1% 22-dihydroergosterol (characterized by RP-HPLC, UV, MS and ^{1}H-NMR), and <1% unidentified component(s). The lathosterol was characterized by the unique C-18 peak at 0.53 ppm in ^{1}H-NMR (its GLC values corresponded to those of 7-dehydrocholesterol, and its RP-HPLC and MS values corresponded to those of cholesterol). The Δ^7-24ξ-methylcholestenol was characterized by the α_c of 1.15 ± 0.02 in RP-HPLC, M^+ of 400 in MS, and the C-18 peak at 0.54 ppm in ^{1}H-NMR. The $\Delta^{7,22}$-24ξ-methylcholestenol was characterized by the α_c of 0.94 ± 0.01 in RP-HPLC, the M^+ of 398 in MS and the C-18 peak at 0.54 ppm in ^{1}H-NMR.

Sterols from HzSNPV. The sterols present in HzSNPV (observed to be a single-nucleocapsid virus by EM), isolated from 38.1 g of infected larvae-fed diet containing cholesterol (Table 1), were 70% cholesterol and 18% sitosterol (characterized by GLC, RP-HPLC, UV, MS and ^{1}H-NMR), 11% 24ξ-methylcholesterol and 2% stigmasterol (characterized by GLC and MS). The latter 24-alkylsterols were probably derived from the plant material in the diet.

The sterols isolated from 120 g of HzSNPV (Sandoz, Inc.) (Table 1) were 56% cholesterol, 12% 24α- and 24β-methylcholesterol, 28% sitosterol and 5% stigmasterol (characterized by GLC, RP-HPLC, UV, MS and ^{1}H-NMR).

The sterols present in HzSNPV isolated from 31.8 g of infected larvae fed a diet containing cholestanol (Table 1) were 99% cholestanol (characterized by GLC, MS and ^{1}H-NMR) and 1% cholesterol (characterized by GLC).

The sterols present in HzSNPV, isolated from 16.6 g of infected larvae-fed diet containing ergosterol (Table 1), were <2% cholesterol, 67% 7-dehydrocholesterol, <2% 22-dihydroergosterol and 30% ergosterol. These sterols were characterized by RP-HPLC and UV.

Quantity of insect tissue sterol associated with HzSNPV. When infected larvae (reared on a diet containing unlabeled cholesterol) were homogenized in a 1:1 ratio with noninfected larvae (reared on a diet containing [7-^{3}H]-cholesterol), the average amount of radioactivity

TABLE 1

Sterol Composition of Infected Larvae of *H. zea* and Their HzSNPV

	% Total sterol in:						
	Infected larvae-fed diet containing:			HzSNPV from larvae-fed diet containing:			HzSNPV
Sterol	Cholestanol	Cholesterol	Ergosterol	Cholestanol	Cholesterol	Ergosterol	(Sandoz Inc.)
Cholestanol	>95	—	—	99	—	—	—
Cholesterol	<5	95	8	1	70	<2	56
7-Dehydrocholesterol	—	—	50	—	—	67	—
Desmosterol	—	<1	—	—	—	—	—
22-Dihydroergosterol	—	—	<1	—	—	<2	—
Ergosterol	—	—	37	—	—	30	—
24ξ-Methylcholesterol	—	<1	—	—	11	—	12
Sitosterol	—	3	—	—	18	—	28
Δ^7-Sterols[a]	—	—	6	—	—	—	—
Stigmasterol	—	<1	—	—	2	—	5
Unidentified	—	—	<1	—	—	—	—

[a]Lathosterol, Δ^7-24ξ-methylcholestenol and $\Delta^{7,22}$-24ξ-methylcholestenol.

associated with the purified nonlabeled polyhedra was 125 ± 115 dpm/10^6 polyhedra. Therefore, the average number of exogenous cholesterol molecules contaminating the polyhedra preparation was $2.63 \times 10^{13} \pm 2.41 \times 10^{13}$ molecules/10^6 polyhedra.

Quantity of sterol in HzSNPV. The average amount of radioactivity associated with polyhedra isolated from larvae (reared on a diet containing [7-^{3}H]cholesterol) was 903 ± 349 dpm/10^6 polyhedra. Therefore, the average number of cholesterol molecules associated with the polyhedra was $9.52 \times 10^{13} \pm 3.7 \times 10^{13}$ molecules/10^6 polyhedra. Consequently, because the percentage of the total cholesterol that was actually associated with the virus, and not due to tissue contamination, was 72.4%, the amount of endogenous cholesterol present in the virus was 6.89×10^{13} molecules/10^6 polyhedra or 40 ng/10^6 polyhedra.

Quantity of sterol in insect tissue. Dietary sterol was present in insect tissue. The average amount of radioactivity present in the fat body from uninfected larvae-fed diet containing [7-^{3}H]cholesterol was 16,104 ± 3,755 dpm/mg tissue. Therefore, the average number of cholesterol molecules in the insect fat body was $1.69 \times 10^{15} \pm 3.9 \times 10^{14}$ molecules/mg of insect tissue or 1.09 μg/mg of insect tissue.

Effect of different host sterols on the virulence of HzSNPV. The effect of various host sterols, on the virulence of HzSNPV obtained from Sandoz, Inc., was determined via bioassays of this virus in larvae-fed diets containing various sterols. The presence of Δ^0-sterol in the tissues of the host did not affect the virulence of the virus because there was no significant difference between the LD_{50}'s of the virus (1,413 and 1,288 polyhedra/larva) in the larvae-fed cholesterol and cholestanol, respectively. Although the larvae-fed ergosterol, and so containing $\Delta^{5,7}$-sterols in their tissues, appeared to be more susceptible to the virus (LD_{50} of 355 polyhedra/larva), this difference may not be significant due to overlapping 95% confidence intervals (Table 2 and Fig. 1).

Effect of different virus sterols on the virulence of HzSNPV. The effect of various viral sterols on the virulence of HzSNPV was determined via bioassays of HzSNPV (propagated in larvae-fed various sterols) in larvae-fed diet containing cholesterol. The presence of Δ^5- versus Δ^0-sterols in the virus affected its virulence because there was a difference in the LD_{50}'s of the viruses (32,359 and 275,423 polyhedra/larva, respectively) in the larvae-fed cholesterol. Because of overlapping 95% confidence intervals (Table 3), the virulence of the virus containing $\Delta^{5,7}$-sterol (LD_{50} of 58,884 polyhedra/larva) may not be different from that of the viruses containing Δ^5- and Δ^0-sterols. However, the HzSNPV propagated in larvae containing the Δ^7-sterol, lathosterol, in their tissues, was the most virulent of all the viruses (LD_{50} of 5,012 polyhedra/larva) (Table 3 and Fig. 2).

The LD_{50} of the laboratory-prepared HzSNPV, containing Δ^5-sterol, was much larger than that of the commercially-obtained HzSNPV (32,359 versus 1,413 polyhedra, respectively). This difference may have occurred because the laboratory virus was always collected from larvae which were moribund, not dead.

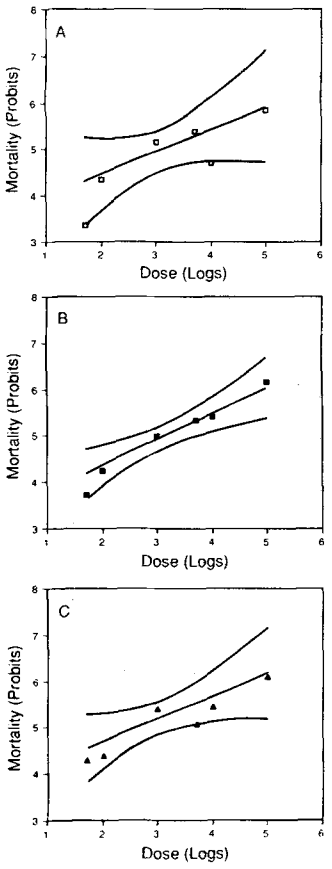

FIG. 1. Dosage-mortality responses of *H. zea* fed cholestanol (A), cholesterol (B) or ergosterol (C) to HzSNPV (Sandoz, Inc.). Fiducial bands represent 95% limits.

TABLE 2

The Dosage-Mortality Responses of *H. zea* Larvae, Fed Various Sterols, to HzSNPV[a]

Sterol in larval diet	No. of larvae in bioassay	LD_{50} (polyhedra/larva)	95% Fiducial limits[b]		Slope	Intercept	X^2	df
			Lower	Upper				
Cholesterol	580	1,413	398	5,495	0.55	3.26	22.19	6[c]
Cholestanol	454	1,288	*	*	0.49	3.47	29.35	4[d]
Ergosterol	376	355	*	2,138	0.48	3.78	13.49	4[e]

[a]Sandoz, Inc.

[b]Asterisks indicate assays in which the heterogeneity was too great to allow calculation of meaningful 95% fiducial limits.

[c]Heterogeneity factor (h) = 3.70.

[d]h = 7.34.

[e]h = 3.37.

TABLE 3

The Dosage-Mortality Responses of *H. zea* Larvae, Fed Diet Containing Cholesterol, to Various HzSNPV

HzSNPV from larvae fed	No. of larvae in bioassay	LD_{50} (polyhedra/larva)	95% Fiducial limits		Slope	Intercept	χ^2	df
			Lower	Upper				
Cholestanol	556	275,423	74,131	3,388,442	0.54	2.05	16.18	6[a]
Ergosterol	424	58,884	19,498	263,027	0.73	1.54	17.21	5[b]
Cholesterol	441	32,359	20,893	51,286	0.72	1.76	7.77	3
Lathosterol	346	5,012	3,631	7,413	1.28	0.28	5.01	4

[a] h = 2.70.
[b] h = 3.44.

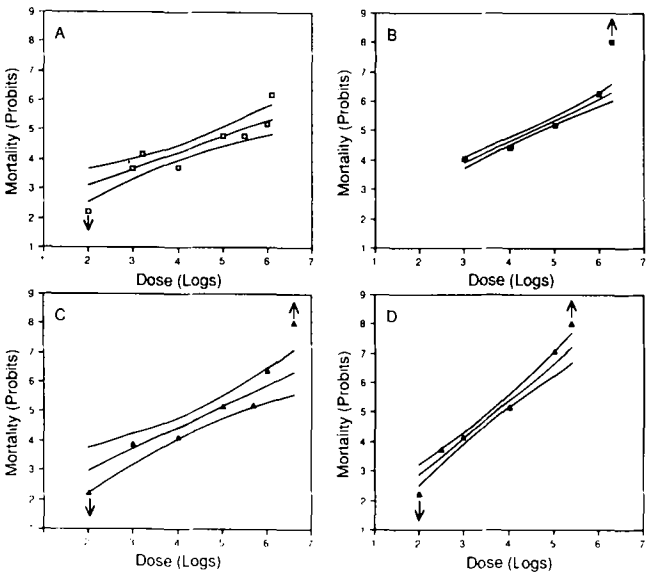

FIG. 2. Dosage-mortality responses of *H. zea*, fed diet containing cholesterol, to HzSNPV isolated from larvae fed cholestanol (A), cholesterol (B), ergosterol (C) or lathosterol (D). Fiducial bands represent 95% limits. Arrows indicate mortality of 0 or 100%, for which the probit value is ∞.

Effect of different virus sterols on the LT_{50} of HzSNPV. The presence of different sterols did not appear to affect the overall course of virus infection because the LT_{50} of the virus in larvae-fed cholestanol, cholesterol, ergosterol or lathosterol ranged from: 6 to 8, 5 to 8, 5 to 7 and 5 to 9 days, respectively.

DISCUSSION

These studies indicate that the sterols present in a host can affect the sterol composition of HzSNPV. When the larvae of *H. zea* contained Δ^5-, Δ^0- or $\Delta^{5,7}$-sterols, primarily, the HzSNPV also contained principally Δ^5-, Δ^0- or $\Delta^{5,7}$-sterols, respectively. This is the first report of an insect or animal virus containing sterols other than cholesterol (11) or desmosterol (6). Only the plant viruses, sonchus yellow net virus (28) and northern cereal mosaic virus (29), are known to contain campesterol, stigmasterol and sitosterol, in addition to cholesterol. (The relative amounts of these phytosterols in the two viruses are: 15 and 13%, 30 and 26%, 34 and 50%, and 21 and 11%, respectively [2].)

Although the major sterols found in the host were the same as those in the virus, some of the minor host sterols (e.g., Δ^7-sterols that were metabolites of ergosterol) were not isolated from the virus. Perhaps the virus selectively utilized certain sterols, which may or may not have been uniformly distributed in the host tissues (e.g., in the membranes), or the amounts of minor sterols in the virus were too small to be detected in this study.

Because the sterols were isolated from whole polyhedra of HzSNPV, it is not known where the molecules were located in the inclusion bodies. However, the envelope associated with the surface of the polyhedra may not be a unit membrane (30) and probably does not contain lipid (31), therefore, the sterol was probably associated with the envelope of the occluded virions. Whether the occluded virion codes for the synthesis of its envelope de novo (32), and then incorporates the host sterols into this structure, or whether it utilizes the host cell membrane (and therefore its sterols) directly, as the non-occluded ones apparently do (33), is not clear.

The results of the present study indicate that the sterol composition of the host not only affects the sterol composition of the virus but also its subsequent infectivity in larvae containing cholesterol as their tissue sterol. The most virulent form of the virus was that isolated from larvae-fed lathosterol followed by the virus isolated from larvae-fed cholesterol, ergosterol and then cholestanol (i.e., the LD_{50} of the virus was 5,012; 32,359; 58,884 and 275,423 polyhedra/larva, respectively). The sterol composition of the virus did not seem to affect the rate of virus replication because there was no significant difference in the LT_{50} of the virus when its sterols were altered. Instead, the sterol composition of the virus may have affected its ability to enter cells, such as midgut cells, via fusion and/or adsorptive endocytosis (viropexis) (32) by changing the fluidity of the envelope (3).

On the other hand, the results of the bioassays in this study indicated that the presence of cholestanol, cholesterol or ergosterol as the principal tissue sterols in the larva, did not affect the infectivity of the virus that was obtained commercially and contained Δ^5-sterols. The slightly higher susceptibility of the larvae-fed ergosterol to the virus (i.e., the LD_{50} of the virus in the larvae-fed cholesterol or cholestanol versus ergosterol was 1,413; 1,288 and 355 polyhedra/larva, respectively) may have been due to the presence of $\Delta^{5,7}$-sterol, per se, in the tissues. However, it is also possible that because the development of uninfected 5th-instar larvae reared on

ergosterol was much slower than on cholesterol and cholestanol (21) (they required 5–6 days longer to reach the 6th instar), such physiologically younger larvae may have been more susceptible to HzSNPV. Ignoffo (34) found that the susceptibility of *H. zea* to NPV decreased as the larvae matured.

The results of this study suggest that if the sterol composition of *H. zea* changed naturally under field conditions (e.g., if the larva consumed a plant with an unusual sterol composition), this might affect the sterol composition and subsequent virulence of HzSNPV. Therefore, further studies on how the sterols of HzSNPV affect the success of this biological control agent in integrated pest management programs are warranted.

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REFERENCES

1. Lenard, J. (1978) *Ann. Rev. Biophys. Bioeng. 7,* 139–165.
2. Jackson, A.O., Franki, R.I.B., Zuidema, D. (1987) in *The Rhabdoviruses* (Wagner, R.R., ed.) pp. 427–508, Plenum Press, New York, NY.
3. Moore, N.F., Patzer, E.J., Shaw, J.M., Thompson, T.E., and Wagner, R.R. (1978) *J. Virol. 27,* 320–329.
4. Pal, R., and Wagner, R.R. (1987) in *The Rhabdoviruses* (Wagner, R.R., ed.) pp. 75–118, Plenum Press, New York, NY.
5. Blough, H.A., and Tiffany, J.M. (1973) *Adv. Lipid Res. 11,* 267–339.
6. Bates, S.R., and Rothblat, G.H. (1972) *J. Virol. 9,* 883–890.
7. Kielian, M.C., and Helenius, A. (1984) *J. Virol. 52,* 281–283.
8. Mooney, J.J., Dalrymple, J.M., Alving, C.R., and Russell, P.K. (1975) *J. Virol. 15,* 225–231.
9. Kundrot, C.E., Spangler, E.A., Kendall, D.A., MacDonald, R.C., and MacDonald, R.I. (1983) *Proc. Natl. Acad. Sci. USA 80,* 1608–1612.
10. Nes, W.R., and McKean, M.L. (1977) in *Biochemistry of Steroids and Other Isopentenoids,* p. 149, University Park Press, Baltimore, MD.
11. Blough, H.A., and Tiffany, J.M. (1980) in *Cell Membranes and Viral Envelopes* (Blough, H.A., and Tiffany, J.M., eds.) Vol. 2, pp. 459–493, Academic Press, New York, NY.
12. Svoboda, J.A., and Thompson, M.J. (1985) in *Comprehensive Insect Physiology Biochemistry and Pharmacology* (Kerkut, G.A., and Gilbert, L.I., eds.) Vol. 10, pp. 137–175, Pergamon Press, New York, NY.
13. Ritter, K.S. (1984) *Arch. Insect Biochem. Physiol. 1,* 281–296.
14. Ritter, K.S. (1986) *Arch. Insect Biochem. Physiol. 3,* 349–362.
15. Balange-Orange, N., and Devauchelle, G. (1982) *Arch. Virol. 73,* 363–367.
16. Bergold, G.H., and Wellington, E.F. (1954) *J. Bacteriol. 67,* 210–216.
17. Wildy, P. (1971) in *Monographs in Virology* (Melnick, J.L., ed.) Vol. 5, p. 32, Karger, New York, NY.
18. Yamamoto, T., and Tanada, Y. (1977) *J. Invertebr. Pathol. 30,* 279–281.
19. Ritter, K.S., and Nes, W.R. (1981) *J. Insect Physiol. 27,* 175–182.
20. Billheimer, J.T., Tavani, D.M., and Ritter, K.S. (1983) *Comp. Biochem. Physiol. 76B,* 127–132.
21. Ritter, K.S., and Nes, W.R. (1981) *J. Insect Physiol. 27,* 419–424.
22. Ritter, K.S., and Tanada, Y. (1978) *Entomophaga 23,* 349–359.
23. Ritter, K.S., Tanada, Y., Hess, R.T., and Omi, E.M. (1982) *J. Invertebr. Pathol. 39,* 203–209.
24. Finney, D.J. (1971) in *Probit Analysis,* 3rd ed., pp. 63–80, Cambridge University Press, New York, NY.
25. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911–917.
26. Ritter, K.S., Weiss, B.A., Norrbom, A.L., and Nes, W.R. (1982) *Comp. Biochem. Physiol. 71B,* 345–349.
27. Nes, W.R., Krevitz, K., Joseph, J., Nes, W.D., Harris, B., Gibbons, G.F., and Patterson, G.W. (1977) *Lipids 12,* 511–527.
28. Selstam, E., and Jackson, A.O. (1983) *J. Gen. Virol. 64,* 1607–1613.
29. Toriyama, S. (1976) *Ann. Phytopathol. Soc. Jpn. 42,* 494–496.
30. Federici, B.A. (1986) in *The Biology of Baculoviruses* (Granados, R.R., and Federici, B.A., eds.) Vol. 1, pp. 61–85, CRC Press, Boca Raton, FL.
31. Minion, F.C., Coons, L.B., and Broome, J.R. (1979) *J. Invertebr. Pathol. 34,* 303–307.
32. Granados, R.R., and Williams, K.A. (1986) in *The Biology of Baculoviruses* (Granados, R.R., and Federici, B.A., eds.) Vol. 1, pp. 89–108, CRC Press, Boca Raton, FL.
33. Volkman, L.E., and Knudson, D.L. (1986) in *The Biology of Baculoviruses* (Granados, R.R., and Federici, B.A., eds.) Vol. 1, pp. 109–127, CRC Press, Boca Raton, FL.
34. Ignoffo, C.M. (1966) *J. Invertebr. Pathol. 8,* 279–282.

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Long Chain Fatty Acid Deficits in Brain Myelin Sphingolipids of Undernourished Rat Pups

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A restricted maternal dietary intake (40% of ad libitum intake) is known to cause myelin deficit that is accompanied by decreased amounts of individual phospholipids and sphingolipids in brain myelin of suckling rats. This communication reports the effects of the same nutritional stress on the fatty acid composition of brain myelin lipids. In myelin of 19-day-old normally fed rats, palmitate (16:0), stearate (18:0) and oleate (18:1) accounted for 80–90% of all fatty acids in phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. Maternal dietary restriction resulted in deficits of total fatty acid content, but did not affect the proportional distribution of individual fatty acids among phospholipids. By contrast, longer chain (22- and 24-carbon) fatty acids accounted for more than half the fatty acid content of myelin cerebroside and sulfatide from the 19-day-old control rat pups. In undernourished rats of that age, proportions of lignocerate (24:0) and nervonate (24:1) in cerebroside and sulfatide were 40–50% lower than those in control rats. This, together with higher proportions of 16:0, 18:0 and 18:1 and a higher ratio of C_{16}–C_{20} to C_{22}–C_{24} in undernourished than in control rats, suggests an impairment in fatty acid chain elongation. Ten days of nutritional rehabilitation failed to restore the fatty acid imbalances; however, after an additional 5 days of ad libitum feeding, the experimental and control values were similar. The undernutrition results in hypomyelination, which is characterized by a proportional decrease in lignoceric and nervonic acids of sphingolipids.
Lipids 23, 1114–1118 (1988).

Postnatal undernutrition induced by various nutritional stresses leads to myelin and lipid deficits in the developing brain (1–8). The severity of the lipid insufficiency depends not only on the type of undernutrition imposed, but also on the time of onset and duration of the stress (3). The maternal dietary intake restriction known to reduce milk production (9) serves as a reliable model for producing consistent growth retardation of offspring (10). A recent study from this laboratory demonstrates that such a dietary restriction caused proportionally similar decreases in amounts of individual phospholipids, spingolipids and cholesterol in the brain myelin of offspring (11). Consequently, the mole ratios of these lipids did not change, suggesting that undernutrition of this type does not selectively affect the accumulation of any particular lipids in myelin. These observations, however, do not exclude the possibility of changes in fatty acid composition of the lipids.

In the mature brain, myelin phospholipids are rich in 16- and 18-carbon fatty acids, whereas sphingolipids contain a greater proportion of 22- and 24-carbon fatty acids (12–15). Earlier studies have shown that the distribution of fatty acids in brain phospholipids and sphingolipids undergoes a marked shift in the presence of severe nutritional insufficiency (3,16). The lipid composition of the brain differs quantitatively and qualitatively from that of myelin (6). Therefore, the changes observed in the brain of undernourished rat pups cannot be extrapolated reliably to myelin. Because a reduction in lipid content and changes in fatty acid composition may lead to hypomyelination (3,17,18), an attempt was made to determine if restriction of maternal dietary intake during the lactating period alters the proportional distribution of fatty acids in the myelin lipids of offspring. The results show substantial reductions in lignocerate (24:0) and nervonate (24:1) in cerebroside and sulfatide, but not in phospholipids.

MATERIALS AND METHODS

Sprague-Dawley rats were used in all experiments. Pregnant rats purchased in early pregnancy (13 to 14 days of gestation) were kept in separate cages and fed a nonpurified diet (Purina Rat Chow, Ralston Purina, St. Louis, MO) ad libitum. Litters of newborn rats were culled to 12 pups each, within 12 hr after birth, and the pups were allowed to be suckled by their dams. All dams were placed in normally fed control or diet-restricted groups. Controls were fed the nonpurified diet ad libitum throughout the lactating period, whereas dams in the experimental group were fed 40% of the same balanced diet consumed by controls until the pups were 19 days of age (11,19). The food intake of the control group was measured daily. The amount of the feed given to the experimental group was adjusted according to feed consumption of the control dams measured on the preceding day.

Undernourished pups were those suckled by dams fed a restricted amount of diet; control pups were suckled by normally fed dams. At the age of 19 days, undernourished pups became hyporeflexive and nearly motionless. Nutritional rehabilitation consisted of providing diets of dams ad libitum when the pups were 19 days old. Control and postnatally undernourished pups were fed the nonpurified diet ad libitum after weaning on day 22.

Myelin was isolated and purified from whole brain according to established sucrose gradient centrifugation procedures (20). The purity and yield of the myelin were determined as described previously (11).

Lipid extraction (21) and chromatographic separation of phospholipids and sphingolipids (22,23) have been described in detail (11). The isolated phospholipids and sphingolipids were transmethylated by the method of Morrison and Smith (24), and the methylated fatty acids were analyzed by gas chromatography (Hewlett-Packard, Model 5880 A, Avondale, PA). Fatty acids were identified by comparing with standards. For measurement of the total fatty acid content in phospholipids, heptadecanoate

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Cr, cerebroside; Su, sulfatide.

was added as an internal standard during transmethylation (25). Weights of fatty acids were calculated by the following equation:

$$\frac{\text{Peak area of fatty acid}}{\text{Peak area of heptadecanoate}} \times \text{weight of heptadecanoate added during transmethylation}$$

Student's *t*-test was used in all statistical comparisons.

RESULTS

Earlier studies demonstrated that undernourished pups produced by the present nutritional manipulation had body weight 60% and brain weight 20% lower than that of controls during the third week of life (11,19). Myelin concentrations of undernourished pups were 60–70% lower than those of normally fed controls (11). The stunted growth was noted as early as 1 week after birth (11). Despite these findings, maternal diets were restricted until the pups were 19 days old in the present study, because at that age active myelination takes place (26), and myelin can be isolated in sufficient quantities for lipid analysis (20).

Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS)—the major components of myelin phospholipids (22)—were analyzed for their fatty acid composition at 19 days of age. In myelin of control pups, palmitic (16:0), stearic (18:0), plus oleic (18:1) acids accounted for 89%, 80% and 78% of total fatty acid in PC, PE and PS, respectively (Table 1). The proportional distributions of the major fatty acids were 16:0 > 18:1 = 18:0 for PC; 18:1 = 18:0 > 16:0 for PE and 18:0 > 18:1 > 16:0 for PS. Relatively lower proportions of arachidonic acid, ranging from 0.6% to 1.3%, were found in the phospholipids. The total longer chain fatty acids (22–24 carbon) accounted for 4.3% in PC, 5.6% in PE and 11.2% in PS. Postnatal undernutrition did not alter the proportional distribution of fatty acid in the three phospholipids. Nevertheless, the total fatty acid content of myelin PC, PE and PS, expressed as μg/g brain tissue, was 50%, 35% and 57% lower, respectively, in undernourished than in control rats.

Unlike the fatty acid profile of phospholipids, longer chain fatty acids (22- and 24-carbon) accounted for 57% of the total fatty acids in myelin cerebroside (Cr) from 19-day-old control rats (Table 2). At that age, undernourished rats had 40% less lignoceric acid (24:0) and 50% less nervonic acid (24:1) than normally fed rat pups. Behenic acid (22:0) was also lower in undernourished than in control rats. The reduction in longer chain fatty acids was accompanied by proportional increase of 16- and 18-carbon fatty acids, resulting in a higher ratio of C_{16}–C_{20} to C_{22}–C_{24} in undernourished, compared with control, rats. The decreased percentages of lignocerate and nervonate and total longer chain fatty acids persisted in postnatally undernourished rats after 10 days of nutritional rehabilitation; however, with an additional 5 days of corrective feeding, the fatty acid composition of Cr in postnatally undernourished rats no longer differed from that of the control group. The sum of C_{22}–C_{24} acids accounted for 55% of the fatty acid content in Cr from 34-day-old rehabilitated rats. This value was similar to

TABLE 1

Fatty Acid Composition of Myelin Phospholipids in 19-Day Undernourished and Normally Fed Rats[a]

	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylserine	
Fatty acid[b]	C[c]	U[d]	C	U	C	U
			% of total fatty acid by weight			
16:0	42.4 ± 2.1	43.8 ± 3.6	12.8 ± 1.1	18.7 ± 4.6	7.3 ± 0.6	8.3 ± 0.7
16:1	1.9 ± 0.3	2.2 ± 0.2	2.9 ± 0.6	2.4 ± 0.8	2.8 ± 0.9	1.4 ± 0.2
18:0	22.2 ± 1.3	20.6 ± 3.3	33.9 ± 3.2	31.2 ± 3.2	50.2 ± 4.5	55.7 ± 5.3
18:1	25.0 ± 1.9	23.1 ± 1.7	33.2 ± 1.3	26.5 ± 3.3	21.1 ± 2.4	17.1 ± 2.2
18:2	0.7 ± 0.2	0.7 ± 0.7	0.9 ± 0.2	1.3 ± 0.7	1.4 ± 0.3	1.0 ± 0.5
18:3	0.1 ± 0.03	0.1 ± 0.03	0.6 ± 0.2	0.3 ± 0.1	0.3 ± 0.05	0.2 ± 0.04
20:0	1.0 ± 0.1	0.8 ± 0.07	1.8 ± 0.1	1.5 ± 0.4	1.3 ± 0.2	1.7 ± 0.3
20:1	1.5 ± 0.1	1.5 ± 0.4	6.4 ± 0.4	4.2 ± 0.8	2.0 ± 0.2	1.2 ± 0.2
20:3	0.3 ± 0.08	0.3 ± 0.09	0.8 ± 0.3	1.2 ± 0.6	1.1 ± 0.3	1.2 ± 0.5
20:4	0.6 ± 0.2	0.8 ± 0.2	1.1 ± 0.6	1.6 ± 0.8	1.3 ± 0.5	0.7 ± 0.2
22:0	1.3 ± 0.3	1.9 ± 0.4	1.9 ± 0.3	3.5 ± 0.7	2.8 ± 1.0	3.5 ± 0.6
22:1	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	1.1 ± 0.6	1.1 ± 0.4	1.2 ± 0.2
22:4	0.9 ± 0.2	0.8 ± 0.3	1.1 ± 0.3	1.4 ± 0.4	1.6 ± 0.6	2.0 ± 0.7
22:6	0.2 ± 0.07	0.4 ± 0.2	0.8 ± 0.3	0.8 ± 0.2	2.3 ± 0.7	1.6 ± 0.6
24:0	1.3 ± 0.2	2.5 ± 0.7	1.3 ± 0.1	4.1 ± 1.6	3.2 ± 0.8	2.9 ± 0.8
24:1	0.2 ± 0.1	0.02 ± 0.01	0.03 ± 0.01	0.1 ± 0.04	0.2 ± 0.06	0.3 ± 0.05
Total fatty acid (μg/g tissue)	100 ± 11	51 ± 2*	55 ± 5	36 ± 4*	51 ± 7	22 ± 4*

[a]Undernutrition was initiated within 12 hr after birth. The values are means ± SEM for 6 samples of isolated myelin. Each sample was prepared from 8 brains of littermates.

[b]Fatty acids are designated by their carbon numbers followed by the number of unsaturated bonds.

[c]C, controls (normally fed).

[d]U, undernourished rat pups.

*Significantly different than the control value at $p < 0.05$.

TABLE 2

Changes in Fatty Acid Composition of Myelin Cerebroside in Undernourished and Rehabilitated Rats[a]

Fatty acids[b]	19-day-old		29-day-old		34-day-old	
	C[c]	U[d]	C	R[e]	C	R
	% of total fatty acid by weight					
16:0	10.2 ± 0.2	20.4 ± 1.4*	12.1 ± 1.7	19.2 ± 1.5*	8.3 ± 0.9	9.6 ± 1.1
16:1	3.3 ± 0.2	6.7 ± 0.2*	2.5 ± 0.3	3.5 ± 0.3	1.7 ± 0.2	2.0 ± 0.2
18:0	12.3 ± 0.3	15.5 ± 0.8*	12.3 ± 0.6	16.9 ± 0.9*	12.6 ± 0.8	12.2 ± 0.4
18:1	8.7 ± 0.3	11.9 ± 0.8*	10.0 ± 0.3	13.7 ± 1.1*	13.2 ± 2.5	11.8 ± 1.5
18:2	3.0 ± 0.2	4.9 ± 0.4*	2.6 ± 0.3	3.9 ± 0.7	3.2 ± 0.3	4.7 ± 0.9
18:3	0.6 ± 0.1	0.7 ± 0.1	0.2 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.2 ± 0.1
20:0	2.4 ± 0.3	1.8 ± 0.2	2.3 ± 0.1	1.4 ± 0.5	2.5 ± 0.1	2.6 ± 0.2
20:1	0.5 ± 0.2	0.3 ± 0.1	0.8 ± 0.2	0.5 ± 0.1	2.2 ± 0.6	0.7 ± 0.2
20:3	0.5 ± 0.1	0.9 ± 0.4	0.3 ± 0.1	0.7 ± 0.1	0.5 ± 0.2	0.2 ± 0.1
20:4	1.6 ± 0.2	1.1 ± 0.2	1.3 ± 0.3	3.0 ± 0.6	0.7 ± 0.2	1.3 ± 0.3
22:0	9.9 ± 0.5	6.6 ± 0.8*	7.6 ± 0.1	7.0 ± 1.1	6.8 ± 0.5	7.0 ± 0.3
22:1	1.9 ± 0.4	1.4 ± 0.4	0.7 ± 0.1	1.1 ± 0.2	0.8 ± 0.2	0.6 ± 0.1
22:4	0.7 ± 0.2	1.6 ± 0.5	0.7 ± 0.1	0.9 ± 0.2	0.3 ± 0.1	0.2 ± 0.1
22:6	1.7 ± 0.2	2.1 ± 0.3	1.4 ± 0.5	2.3 ± 0.6	0.4 ± 0.2	0.5 ± 0.2
24:0	28.7 ± 0.7	17.2 ± 1.1*	24.4 ± 2.1	15.0 ± 0.4*	22.8 ± 1.0	25.2 ± 2.4
24:1	14.0 ± 0.7	6.9 ± 0.8*	20.8 ± 0.6	10.3 ± 1.5*	23.4 ± 1.6	21.2 ± 1.4
$\frac{C_{16}-C_{20}}{C_{22}-C_{24}}$	0.74 ± 0.1	1.80 ± 0.3*	0.82 ± 0.1	1.93 ± 0.2	0.83 ± 0.1	0.83 ± 0.1

[a] Undernutrition was initiated within 12 hr after birth. Rehabilitation was begun after 19 days of undernutrition and continued until pups reached the indicated age. See Materials and Methods for details of the rehabilitation regimen. The values are means ± SEM for 6 samples of isolated myelin. Each sample was prepared from 8 (19-day-old) or 3 (29- and 34-day-old) brains of littermates.

[b] Fatty acids are designated by their carbon numbers followed by the number of unsaturated bonds.

[c] C, controls (normally fed).

[d] U, undernourished rat pups.

[e] R, rehabilitated rat pups.

*Significantly different than the control value at $p < .005$.

that of control rats. Thus, extended nutritional rehabilitation yielded a C_{16}-C_{20} to C_{22}-C_{24} ratio that was comparable with the ratio for normally fed rats.

The fatty acid composition of myelin sulfatide (Su) resembled that of Cr in 19-day-old normally fed animals (Tables 2 and 3). Su contained a high percentage (56%) of total fatty acids as C_{22}-C_{24} with a C_{16}-C_{20} to C_{22}-C_{24} ratio of 0.77 (Table 3). Postnatally undernourished rats had reduced lignocerate and nervonate percentages with a concomitant increase in palmitate, stearate and oleate. Despite nutritional rehabilitation for 10 days, the deficits of lignocerate and nervonate persisted; when combined with the high percentages of palmitate, stearate and oleate they resulted in a higher ratio of C_{16}-C_{20} to C_{22}-C_{24} than was found in the control group (1.82 vs 0.77). Extending the period of nutritional rehabilitation to 15 days (i.e., until 34 days of age) corrected the deficits in longer chain fatty acids and restored the fatty acid profile to normal.

DISCUSSION

PC, PE and PS are the dominant phospholipids in purified myelin isolated from rat pups (11). The analysis of the fatty acid composition of myelin from normally fed pups disclosed that palmitate, stearate and oleate constitute 80–90% of all fatty acids in PC, PE and PS, consistent with findings reported by Fishman et al. (13) for rat brain and by O'Brien and Sampson (12) for human brain. Arachidonate is a minor component of myelin phospholipids, accounting for less than 2% of total fatty acids. Fishman et al. (13) have shown that undernutrition induced by increasing the litter size does not alter the fatty acid composition of myelin PC and PE in rat pups during the first 21 days of life. Similarly, restriction of maternal dietary intake in the present study did not affect the fatty acid composition of myelin PC, PE and PS in offspring. By contrast, undernutrition due to an increased litter size was associated with decreased proportions of arachidonate and polyunsaturated fatty acids in brain phospholipid in a study by Srinivasa Rao (16). One explanation for the different reported effects of undernutrition on fatty acid composition is that in the latter study total phospholipids instead of individual phospholipids were analyzed and brain tissue rather than myelin was used for extraction of phospholipids. It should be stressed that the phospholipid compositions of brain tissue and myelin differ widely in normally fed rats (13,16,27). In particular, brain phospholipids contain five- to eightfold more arachidonic acid than do myelin lipids (13,16,27). Hence, if one expects to identify subtle effects of undernutrition on myelin development, the analysis should be directed to the phospholipids of myelin and not to those of brain.

TABLE 3

Changes in Fatty Acid Composition of Myelin Sulfatide in Undernourished and Rehabilitated Rats[a]

Fatty acids[b]	19-day-old		29-day-old		34-day-old	
	C[c]	U[d]	C	R[e]	C	R
	% of total fatty acid by weight					
16:0	13.5 ± 1.7	21.4 ± 0.4*	12.2 ± 1.7	20.7 ± 2.4*	13.4 ± 1.0	13.0 ± 3.1
16:1	3.5 ± 0.9	2.1 ± 0.4	2.5 ± 0.3	3.4 ± 0.3	1.5 ± 0.8	2.4 ± 0.6
18:0	13.1 ± 1.1	18.1 ± 1.2*	12.4 ± 0.7	17.7 ± 1.7*	19.8 ± 0.8	19.2 ± 1.8
18:1	4.3 ± 0.6	7.5 ± 1.0*	0.8 ± 0.3	15.1 ± 1.6*	8.6 ± 0.6	8.9 ± 1.1
18:2	3.0 ± 0.1	3.3 ± 0.6	2.6 ± 0.2	4.1 ± 1.1	2.7 ± 0.1	3.5 ± 0.2
18:3	1.4 ± 0.4	1.9 ± 0.4	0.3 ± 0.1	0.9 ± 0.3	2.1 ± 0.2	3.2 ± 0.5
20:0	3.7 ± 0.7	3.7 ± 0.5	2.4 ± 0.1	1.6 ± 0.3	2.5 ± 0.1	2.1 ± 0.2
20:1	0.4 ± 0.1	2.6 ± 0.3*	0.9 ± 0.3	0.6 ± 0.1	0.9 ± 0.4	1.6 ± 0.8
20:3	0.6 ± 0.1	1.1 ± 0.2	0.3 ± 0.1	0.5 ± 0.2	0.3 ± 0.1	0.7 ± 0.3
20:4	0.2 ± 0.1	1.1 ± 0.4	1.3 ± 0.4	2.9 ± 0.8	1.2 ± 0.3	2.1 ± 0.9
22:0	10.4 ± 1.1	8.7 ± 1.0	7.9 ± 0.2	4.3 ± 1.0*	7.8 ± 0.6	6.9 ± 0.6
22:1	3.0 ± 0.4	4.4 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	0.6 ± 0.1	0.8 ± 0.2
22:4	2.3 ± 0.2	4.9 ± 1.1	0.6 ± 0.2	0.8 ± 0.1	1.1 ± 0.2	1.3 ± 0.1
22:6	1.6 ± 0.2	2.2 ± 0.4	1.2 ± 0.3	1.7 ± 0.6	1.4 ± 0.1	1.2 ± 0.1
24:0	25.8 ± 2.7	9.4 ± 2.0*	24.8 ± 2.2	14.5 ± 2.0*	22.5 ± 1.5	20.7 ± 0.5
24:1	13.2 ± 0.8	7.6 ± 1.2*	20.1 ± 0.9	10.4 ± 1.8*	13.6 ± 1.2	12.5 ± 1.9
$\frac{C_{16}-C_{20}}{C_{22}-C_{24}}$	0.77 ± 0.2	1.69 ± 0.3*	0.77 ± 0.1	1.82 ± 0.3*	1.13 ± 0.2	1.25 ± 0.1

[a] Undernutrition was initiated within 12 hr after birth. Rehabilitation was begun after 19 days of undernutrition and continued until pups reached the indicated age. See Materials and Methods for details of the rehabilitation regimen. Values are means ± SEM for 6 samples of isolated myelin. The samples were prepared from the same animals described in Table 2.

[b] Fatty acids are designated by their carbon numbers followed by the number of unsaturated bonds.

[c] C, controls (normally fed).

[d] U, undernourished rat pups.

[e] R, rehabilitated rat pups.

*Significantly different from the control value at $p < 0.05$.

The most important finding of the present study is that undernutrition imposed for 19 days sharply reduces the proportional distribution of lignocerate and nervonate in the myelin-specific cerebroside and sulfatide. As a result, the ratio of C_{16}-C_{20} to C_{22}-C_{24} was higher in myelin from undernourished rats than from that of the controls. This contrasts with the unaltered fatty acid composition of Cr and Su found by Fishman et al. (13) in large litters at 21 days of age. However, in their study, deficits of lignocerate and nervonate were apparent if the postnatal undernutrition produced by larger litters was continued through the postweaning period. One interpretation of these variable results is that different nutritional stresses affect myelin lipid in different ways depending on the duration of the stress (3,5). When increased litter size was combined with a low protein diet after weaning to produce undernutrition, Srinivasa Rao (16) observed reductions of lignocerate and nervonate of cerebroside in brain. Because cerebroside, a myelin-specific lipid, is found exclusively in myelin (6,26), it is reasonable to assume that cerebroside analyzed in the brain by this investigator could represent myelin lipids. Nevertheless, this is consistent with the present study showing that changes in fatty acid composition by postnatal undernutrition is specific for sphingolipids.

The metabolic activity of myelin lipid, such as its membrane-stabilizing action, depends on fatty acid composition (12,28). Increased amounts of longer chain fatty acids, particularly 24:0 and 24:1 in cerebroside and sulfatide (12,15), coincide with periods of rapid synthesis (23) and accumulation (29) of cerebroside and sulfatide during brain maturation. Thus, deficits of 24:0 and 24:1 in sphingolipids (Tables 2 and 3), together with the insufficiency of cerebroside and sulfatide (11), may be important contributing factors of the hypomyelination commonly observed in undernourished pups (3,17,30).

Whether the reduced proportions of 24:0 and 24:1 in cerebroside and sulfatide reflect lower amounts of these fatty acids in myelin remains uncertain. Nonetheless, the 50–60% reduction in concentrations of cerebroside and sulfatide (11) are likely to reduce the amounts of 24:0 and 24:1. In fact, this notion is strongly supported by the observation that myelin of undernourished rat pups had a reduced amount of fatty acids of phospholipids (Table 1), primarily due to low concentration of the lipids (11). Brain shorter chain fatty acids (e.g., 16- and 18-carbon acids) are derived from de novo synthesis and diet (31,33), whereas longer chain fatty acids (e.g., 22- and 24-carbon acids) arise from chain elongation (32–34). Hence, the deficits of 24:0 and 24:1 observed in postnatally undernourished rat pups are best explained by an impairment in fatty acid chain elongation, consistent with the higher ratios of C_{16}-C_{20} to C_{22}-C_{24} in undernourished vs control rats.

Not 10 but 15 days of nutritional rehabilitation by ad libitum feeding corrected the 24:0 and 24:1 deficits in

both cerebroside and sulfatide. The same regimen also restored concentrations of cerebroside and sulfatide to levels comparable with those in normally fed rats (11). The reversibility of these compositional changes suggests that timely nutritional intervention can prevent permanent biochemical lesion of the brain in offspring that are poorly nourished during lactation period.

Lignocerate (24:0) and nervonate (24:1) are the marker fatty acids of brain myelin (16) and the increases in the myelin content of these nonhydroxy fatty acids coincide with brain maturation and development (28). However, it should be noted that, although 24:0 and 24:1 constitute the major proportion of the longer chain fatty acids in sphingolipids (28), these lipids also contain α-hydroxy fatty acids (15,35). Undernutrition induced by increasing the litter size has been shown to increase the amount of 24:1 hydroxy fatty acid in Cr (13), whereas that induced by feeding a low protein diet reduced the 24:1 hydroxy fatty acid content but had no effect on 24:0 hydroxy fatty acid content in Cr (16). A further investigation on the effects of the present model of undernutrition on the α-hydroxy fatty acid composition in myelin sphingolipids is warranted.

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REFERENCES

1. Dobbing, J. (1964) *Proc. Roy. Soc. Biol. 159,* 503-509.
2. Dobbing, J., and Widdowson, E.M. (1965) *Brain 88,* 357-366.
3. Krigman, M.R., and Hogan, E.L. (1976) *Brain Res. 107,* 239-255.
4. Simons, S.D., and Johnston, P.V. (1976) *J. Neurochem. 27,* 63-69.
5. Yusuf, H.K.M., Haque, Z., and Mozaffar, Z. (1981) *J. Neurochem. 36,* 924-930.
6. Wiggins, R.C. (1982) *Brain Res. Rev. 4,* 151-175.
7. Fuller, G.N., Johnston, D.A., and Wiggins, R.C. (1984) *Brain Res. 290,* 195-198.
8. Vitiello, R., Di Benedetta, C., and Gombos, G. (1985) *Biochem. Soc. Trans. 13,* 758-759.
9. Grigor, M.R., Allan, J.E., Carrington, J.M., Carne, A., Geursen, A., Young, D., Thompson, M.P., Haynes, E.B., and Coleman, R.A. (1987) *J. Nutr. 117,* 1247-1258.
10. Altman, J., Das, G.D., Sudarshan, K., and Anderson, J.B. (1971) *Develop. Psychobiol. 4,* 55-70.
11. Yeh, Y-Y. (1988) *J. Neurosci. Res. 19,* 357-363.
12. O'Brien, J.S., and Sampson, E.L. (1964) *J. Lipid Res. 6,* 545-551.
13. Fishman, M.A., Madyastha, P., and Prensky, A.L. (1971) *Lipids 6,* 458-465.
14. Svennerholm, L. (1968) *J. Lipid Res. 9,* 570-579.
15. Svennerholm, L., and Stallberg-Stenhagen, S. (1968) *J. Lipid Res. 9,* 215-225.
16. Srinivasa Rao, P. (1979) *Nutr. Metabol. 23,* 136-144.
17. Davison, A.N., and Dobbing, J. (1966) *Br. Med. Bull. 22,* 40-44.
18. Wiggins, R.C., Miller, S.L., Benjamins, J.A., Krigman, M.R., and Morell, P. (1976) *Brain Res. 107,* 257-273.
19. Yeh, Y-Y., Zee, P., Owen, G.L., and Walker, E. (1982) *J. Nutr. 112,* 1162-1168.
20. Norton, W.T., and Poduslo, S.E. (1973) *J. Neurochem. 21,* 749-757.
21. Folch, J., Lees, M., and Sloan-Stanley, G.H. (1957) *J. Biol. Chem. 226,* 497-509.
22. Norton, W.T., and Autilio, L.A. (1966) *J. Neurochem. 13,* 213-222.
23. Yeh, Y-Y. (1984) *J. Neurosci. Res. 11,* 383-394.
24. Morrison, W.R., and Smith, L.M. (1964) *J. Lipid Res. 5,* 600-608.
25. Tinoco, J., Shannon, A., Miljanich, P., Lyman, R.L., and Okey, R. (1962) *Anal. Biochem. 3,* 514-520.
26. Norton, W.T., and Poduslo, S.E. (1973) *J. Neurochem. 21,* 759-773.
27. Crawford, C.G., and Wells, M.A. (1979) *Lipids 14,* 757-762.
28. Srinivasa Rao, P. (1977) *Lipids 12,* 335-339.
29. Wells, M.A., and Dittmer, J.C. (1967) *Biochemistry 6,* 3169-3175.
30. Wiggins, R.C., and Fuller, G.N. (1978) *J. Neurochem. 30,* 1231-1237.
31. Dhopeshwarker, G.A., Subramann, C., and Mead, F. (1973) *Biochim. Biophys. Acta 296,* 257-264.
32. Murad, S., and Kishimoto, Y. (1978) *Arch. Biochem. Biophys. 185,* 300-306.
33. Aeberhard, E., and Menkes, J.H. (1968) *J. Biol. Chem. 243,* 3834-3840.
34. Bourre, J.M., Paturneau-Jouas, M.Y., Dandu, O.L., and Baumann, N.A. (1977) *Eur. J. Biochem. 72,* 41-47.
35. Kishimoto, Y., and Radin, N.S. (1959) *J. Lipid Res. 1,* 79-82.

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A Novel Bioaction of PAF: Induction of Microbicidal Activity in Guinea Pig Bone Marrow Cells

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When guinea pig bone marrow cells were incubated in the presence of 10^{-8} to 10^{-6} M platelet activating factor (PAF) for 24 to 72 hr, microbicidal activity against *Candida parapsilosis* of cells was augmented. This augmentation was inhibited by PAF-specific antagonists, CV6209 or FR900452. PAF-specific binding sites with a high affinity were found on guinea pig bone marrow cells. Carrageenan or 2-chloroadenosine, reagents known to be preferentially cytotoxic to macrophages, abolished the microbicidal activity of PAF-treated bone marrow cells. Macrophages prepared from the peritoneal cavity, however, acquired no appreciable microbicidal action by treatment with PAF. These observations suggest that PAF may affect a class of guinea pig bone marrow cells through binding to receptors specific to PAF, resulting in activation and/or induction of differentiation of monocyte-macrophage lineage cells.
Lipids 23, 1119-1124 (1988).

Platelet activating factor (PAF) was first identified as a potent platelet aggregating agent released from antigen-stimulated, IgE-sensitized rabbit basophils (1). It was later shown to be generated, when appropriately stimulated, by various inflammatory cells (2) such as monocytes (3), macrophages (4), neutrophils (5), eosinophils (6), platelets (7) and endothelial cells (8). It was also revealed that PAF-induced hypotension (9), neutrophil activation (10), monocyte aggregation (11), macrophage activation (12), eosinophil chemotaxis (13), smooth muscle constraction (14) and vascular permeability increased (15).

Besides being involved as a potent mediator in various pathological and physiological reactions, evidence recently accumulated suggests that PAF is a component in the regulation of cellular immune responses (16). It was shown that, when PAF was added to human peripheral blood lymphocyte cultures stimulated with mitogen, lymphocyte proliferation was inhibited (17). Natural killer cell-mediated lysis of leukemia cells was revealed to be enhanced by picomolar concentrations of PAF (18). Berdel et al. (19) showed that intravenous injection of bone marrow macrophages pretreated with a weak PAF agonist, ET18-OMe (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine), inhibited the development of metastasis of 3-Lewis lung carcinoma in C57B1/6 mice. We recently reported that the antitumor activity of synthetic alkyl-ether lipid analogs might be partly by activation of bone marrow macrophages in vivo, and that the activation is by their binding to PAF receptors on these cells (20). For the present paper, we examined the effect of PAF on guinea pig bone marrow cells and found that PAF induced candidacidal activity.

MATERIALS AND METHODS

Chemicals. Fetal bovine serum was obtained from Hy Clone Laboratories, Logan, UT. RPMI 1640 Medium was from MA Bioproducts, Walkerville, MD. K-Carrageenan was obtained from Sigma Chemical Co., St. Louis, MO.

PAF, PAF agonists and PAF antagonists. PAF, PAF agonists (12) and PAF antagonists CV6209 (21) used in the present study were provided by Takeda Chemical Ind. Ltd. (Osaka, Japan). Their structures are listed in Table 1. A PAF antagonist FR900452 (22) was provided by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). [^{3}H]-PAF was obtained from Amersham (Buckinghamshire, U.K.).

Animals and microorganisms. Female Hartley guinea pigs (body weight 250-500 g) were obtained from the Japan Bio-supply, Tokyo, Japan. *Candida parapsilosis,* isolated and identified in the laboratory of one of the authors (23,24), was used for a microbicidal test. They were cultured on Sabouraud dextrose agar plates.

Preparation of bone marrow cells. Bone marrow cells were prepared from femurs of guinea pigs. After exsanguination, femurs were cut at one end and bone marrow cells were flushed out by injecting 5 ml of Hank's balanced salt solution into the end. After standing in ice for 5 min, the supernatant was collected and centrifuged at 150 × *g* for 5 min. Sedimented cells were hemolyzed by adding 10 ml lysing buffer (140 mM NH_4Cl, 17 mM Tris-HCl, pH 7.2) and incubated for 5 min at 37°C. Cells were then washed with RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 μg/ml).

Assay method for microbicidal activity of bone marrow cells. Microbicidal activity was determined by the methods previously described (24). In brief, bone marrow cells (1×10^5 cells) were seeded in each well of flat-bottomed 96-well plates (Falcon 3072) with 0.2 ml of the RPMI 1640 medium with 10% fetal bovine serum. After 1 to 2 days incubation at 37°C under 5% CO_2 atmosphere, cells were treated with PAF or its agonists for 72 hr, and then infected with 10 μl of 2-fold serially diluted *C. parapsilosis* suspensions. The number of microbes present in the suspension was initially determined by subcultivation onto Sabouraud's agar. In subsequent experiments, turbidity was routinely monitored by measuring the absorbance at 650 nm, because there was good correlation between turbidity and cell number. After incubation for 48 hr at 37°C in a 5% CO_2 incubator, outgrowth of candida cells in each well was determined under a microscope. Microbicidal activity of bone marrow cells (the maximum number of microbes killed [MNMK]) was evaluated as the maximum number of

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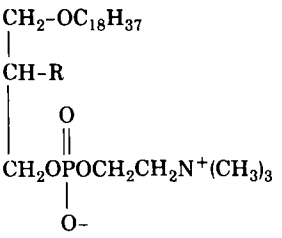
Abbreviations: MNMK, maximum number of microbes killed; PAF, platelet activating factor.

TABLE 1

Structures of PAF, PAF Agonists and PAF Antagonists



Systemic names	Abbreviation	Structure R
1-*O*-Octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine	PAF	$-OCOCH_3$
1-*O*-Octadecyl-2-*O*-(*N,N*-dimethyl-carbamoyl)-*sn*-glycero-3-phosphocholine	Compound I	$-OCON(CH_3)_2$
3-*O*-Octadecyl-2-*O*-(*N,N*-dimethyl-carbamoyl)-*sn*-glycero-1-phosphocholine	Compound II	$-OCON(CH_3)_2$
racemic 1-*O*-Octadecyl-2-*O*-methyl-glycero-3-phosphocholine	Compound III	$-OCH_3$
1-*O*-Octadecyl-*sn*-glycero-3-phosphocholine	lysoPAF	$-OH$

candida was added to the wells where the growth was completely suppressed. The relative increase of MNMK in the treated sample compared with the control was also calculated as follows: Activation Index = $\log_2$ (MNMK of treated sample/MNMK of control sample).

Quantitation of PAF binding. To a 200 μl of bone marrow cell suspension (1.25×10^7 cells per ml) in calcium- and magnesium-free Hank's balanced salt solution with 0.1% bovine serum albumin (binding buffer) in an Eppendorf tube (1.5 ml), a 50 μl radioactive PAF solution with or without a 1000-fold molar excess of unlabeled PAF was added. After incubation at 22°C for 5 min, a 200 μl cell suspension was filtered through a Whatman GF/C glass-fiber filter with a vacuum apparatus (Saltorius, SM16547) attached to an aspirator. Each filter was washed quickly with 5 ml ice-cold binding buffer four times and dried. Radioactivity bound on the filter was then measured.

TABLE 2

Induction of Candidacidal Activity of Guinea Pig Bone Marrow Cells by PAF

Treatment	Maximum numbers of microbes killed			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
PAF 10^{-6} M	2350	2680	2180	>5360
10^{-7} M	1180	670	1090	5360
10^{-8} M	147	670	34	670
Control (0.25% BSA)	37	42	9	42

Bone marrow cells seeded at 10^5 cells per well were cultured for 2 days before treatment. They were incubated for 3 days in the presence of PAF and infected with *C. parapsilosis*. After 2 days incubation, MNMK was determined as described in Materials and Methods.

RESULTS

Effect of PAF on candidacidal activity of cultured bone marrow cells. Guinea pig bone marrow cells were placed in each well of 96-well plates and were examined for candidacidal activity. When 10^{-8} to 10^{-6} M PAF was added 3 days prior to infection with *C. parapsilosis*, the MNMK values were significantly larger than those observed with control culture (Table 2). The samples in wells, where microbes were not microscopically found, were properly diluted and subcultured onto the agar. No colony formation was observed, which suggests that no viable microbes remained.

In addition to PAF, synthetic PAF agonists, Compounds I and III, both of which were resistant to deacetylation (12), also showed appreciable activity (Fig. 1). Compound I was more active than PAF; the effective dose of Compound I ranged between 1/100 to 1/1,000 of that of PAF. Compound III showed only weak activity, and lysoPAF showed no appreciable activity (data not shown).

This candidacidal activity increased during incubation of cells with a PAF agonist, before microbial infection; MNMK values of bone marrow cells incubated with 10^{-7} M of Compound I for 1 and 2 days prior to microbial infection were 400 and 1,450, respectively. No significant induction of candidacidal activity was observed when Compound I and the microbe were simultaneously added.

Induction of candidacidal activity through specific PAF binding sites. Compound I has the same configuration as natural PAF. The induction of candidacidal activity was stereospecific; Compound II, an *sn*-1 enantiomer of Compound I, induced no appreciable microbicidal activity (Fig. 1). An enantiomer of PAF did not induce any activity even at 10^{-5} M (data not shown). Specific PAF antagonists, CV6209 and FR900452, suppressed the augmentation of candidacidal activity induced by 10^{-6} M PAF in a dose-dependent manner (Fig. 2).

Guinea pig bone marrow cells were examined to see if they carry specific binding sites for PAF. A total amount of radioactive PAF bound to the cells increased when

greater quantities of radioactive PAF were added to the cell suspension. Nonspecific binding, the binding of radioactive PAF in the presence of a 1,000-fold molar excess of unlabeled PAF, was not saturable, whereas specific binding (the total binding minus nonspecific binding) seemed saturable (Fig. 3A). Two distinct binding sites for PAF were tentatively determined by Scatchard plot analysis of the binding (Fig. 3B). One site exhibited a high affinity with a Kd = 1.1×10^{-10} M and a capacity of 8.8×10^3 binding sites per bone marrow cell (mean, n = 3). The other site, with a substantially lower affinity and greater binding capacity, probably corresponds to nonreceptor uptake of PAF into the lipid bilayer.

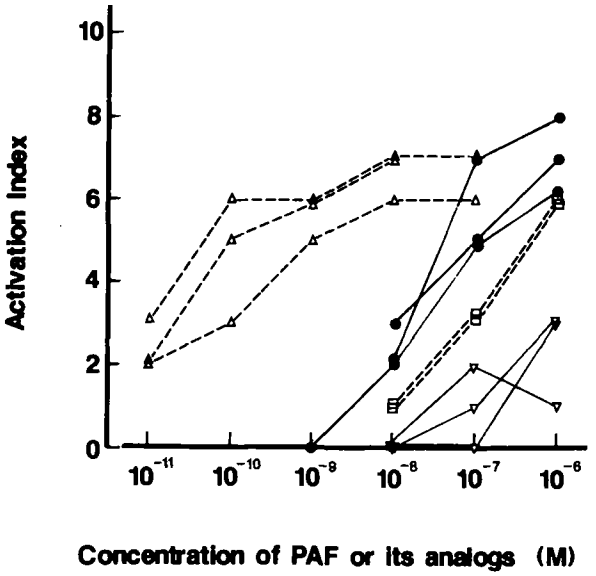


FIG. 1. Induction of candidacidal activity of guinea pig bone marrow cells by PAF (●) and its agonists, Compound I (△), Compound II (▽) and Compound III (□). The experiments were carried out as described in Table 2. Activation Index was determined as described in Materials and Methods. Each line represents experimental result independently performed.

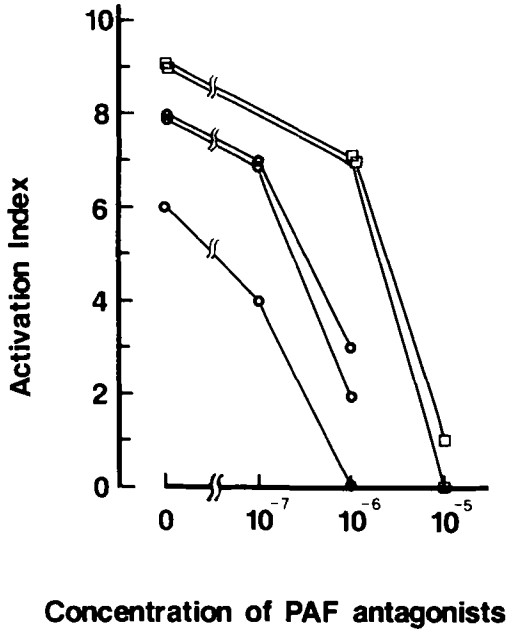


FIG. 2. Effect of antagonists on PAF-induced candidacidal activity of bone marrow cells. Candidacidal activity of bone marrow cells was induced by 10^{-6} M PAF as described in Table 2. PAF antagonists, CV6209 (○) or FR900452 (□), were added to the culture 30 min prior to treatment with PAF and were present throughout the experiment. Each line represents experimental result independently performed.

Possible effector cells in the candidacidal action. It was of great interest to clarify what kind of bone marrow cells were responsible for the microbicidal action. When cultivated without PAF in vitro, the number of viable bone marrow cells present in each well decreased gradually. Addition of 10^{-6} M PAF into the culture medium significantly restored or even increased the number of viable cells (Fig. 4). These observations may suggest that PAF induced prolification of some bone marrow cells.

It was shown that carrageenan taken up by macrophages in vitro (25) was cytotoxic to them (26). On the other hand, either the number or bacteriocidal activity of polymorphonuclear cells was not significantly impaired by carrageenan (27). Bone marrow cells cultured in the presence of PAF were treated with carrageenan prior to microbial infection and examined for the effect against candida. Treatment with carrageenan abolished the induction of candidacidal activity (Table 3).

The inhibitory effect was also observed with 2-chloroadenosine (Table 3), which also was reported to be specifically cytotoxic to macrophages (28). These findings indicated that effector cells for candidacidal activity might belong to monocyte-macrophage lineage cells.

It should be noted here that mature peritoneal macrophages derived from guinea pig injected with mineral oil acquire no appreciable candidacidal activity when treated in vitro with PAF or PAF agonists (Table 4).

DISCUSSION

Guinea pig bone marrow cells were treated with PAF and were examined for action on *C. parapsilosis*. Compared

TABLE 3

Effect of Carrageenan and 2-Chloroadenosine on PAF-induced Candidacidal Activity of Bone Marrow Cells

	Maximum number of microbes killed			
	72 hr incubation		3 hr incubation	
Treatment	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Control (0.25% BSA)	<8	164	<8	<8
PAF 10^{-5} M	4080	5350	4080	4720
PAF 10^{-5} M, K-carrageenan 0.1 mg/ml	64	<20	128	128
K-Carrageenan 0.1 mg/ml	<8	<20	<8	<8
PAF 10^{-5} M, 2-chloroadenosine 0.1 mM	<8	<20	<8	<8
2-Chloroadenosine 0.1 mM	<8	<20	<8	<8

Experiments were carried out essentially identically to that described in Table 2. Bone marrow cells were treated with PAF (10^{-5} M) for 72 hr before *C. parapsilosis* infection. In Exp. 1 and 2, drugs were added to the culture simultaneously with PAF. In Exp. 3 and 4, drugs were added to the culture 3 hr prior to the infection.

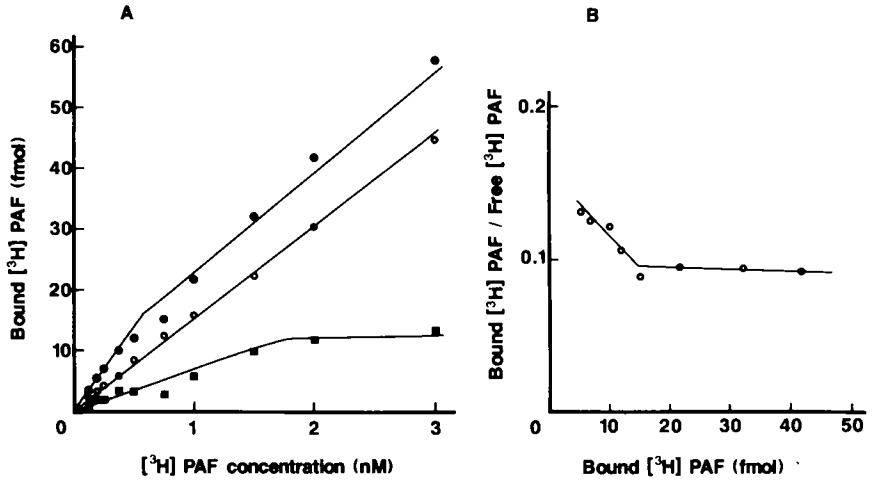


FIG. 3. Binding of [^{3}H]PAF to guinea pig bone marrow cells. A) Bone marrow cells were used for binding assay on the any of collection. Experiments were performed as described in Materials and Methods. Total binding, ●; nonspecific binding, ○; specific binding, ■. Each point is the mean of triplicates and standard deviation is less than 10% of the counts. Under conditions employed, no appreciable PAF degradation was observed. B) Scatchard plot of specific [^{3}H]PAF binding data.

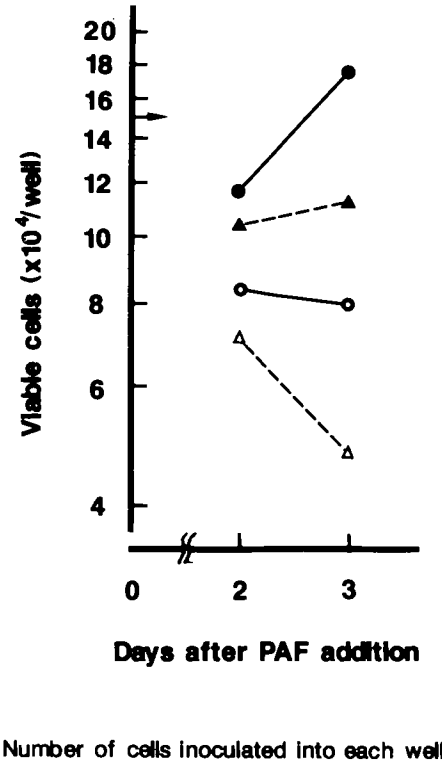


FIG. 4. Time course of the number of viable cells incubated in the presence or absence of PAF. Bone marrow cells were seeded at 1.5 × 10^6 cells per dish (30 mm diameter) 24 hr prior to addition of PAF. After 2 or 3 days incubation with (●▲) or without (○△) 10^{-5} M PAF, number of viable cells were calculated by dye exclusion method. Results of two independent experiments (Exp. 1, ○●; Exp. 2, △▲) were shown.

TABLE 4

Effect of PAF and Its Agonists on Candidacidal Activity of Guinea Pig Peritoneal Macrophages

Treatment		Maximum numbers of microbes killed		
		Exp. 1	Exp. 2	Exp. 3
PAF	10^{-6} M	19	168	
	10^{-7} M	19	168	
Compound I	10^{-6} M			88
	10^{-7} M			44
Compound III	10^{-6} M			22
	10^{-7} M			44
Control (0.25% BSA)		38	83	44

Peritoneal macrophages were prepared as adherent cells from peritoneal exudated cells harvested from guinea pig treated with mineral oil and were cultured with either PAF or its agonists for 24 hr. They were then infected with *C. parapsilosis*, and MNMK was determined after 2 days incubation as described in Materials and Methods.

to nontreated cells, PAF-treated cells caused growth inhibition of candida. The effect of PAF-treated bone marrow cells on candida was not cytostatic but cytocidal, because no viable microbes were detected in wells where microbes were incubated with PAF-treated bone marrow cells.

Among PAF agonists tested, Compound I had the most potent effect on bone marrow cells. The relative activity of compounds to induce candidacidal activity was similar to their relative potency to activate peritoneal macrophages of guinea pig in vitro (12). Unlike the activity to platelets, the activity of PAF to induce candidacidal activity in bone marrow cells, as well as the activity to augment glucose consumption in macrophages (12), was rather poor as compared with Compound I. However, Compound I, opposite to PAF, is resistant to metabolic inactivation (29). Poor activity of PAF toward both bone marrow cells and peritoneal macrophages could be due to its rapid degradation to its inactive form, lysoPAF, by a potent acetylhydrolase in serum, because

a relatively longer period of incubation of cells with PAF was required for showing these activities.

The similarity of the present structure-activity relationship to that observed with macrophage activation, together with inhibition by PAF antagonists of inducing augmentation of candidacidal activity, suggests that the activity may be through a specific mechanism via binding to receptors on some cell populations in bone marrow. Consistently, specific binding sites for PAF were detected on bone marrow cells. They showed high affinity and were saturable.

Longer incubation of bone marrow cells after addition of PAF caused more effective induction of candidacidal activity. On the other hand, relatively brief stimulation with PAF might be sufficient to induce microbicidal activity because of a rapid degradation of PAF in fetal bovine serum containing medium. The total number of viable cells observed in the incubation mixture containing PAF was significantly larger than that of control cultivation. The effect of PAF could not be due simply to an activation of cells preexisting in bone marrow but, at least partly, due to induction of prolification and/or differentiation of immature cells. It was previously reported that Compound III induced in vitro cell differentiation of human (HL-60) and mouse (M1) myeloid cells (30). Unlike the induction of candidacidal activity by PAF agonists, the induction of differentiation of M1 cell was not stereospecific; both *sn*-3 and *sn*-1 enantiomers of Compound III were found to equally induce M1 cell differentiation. Differentiation of M1 cells induced by Compound III may be through a different mechanism than that involved in activation of bone marrow cells.

Effector cells responsible for killing candida are either polymorphonuclear leukocytes or monocyte-macrophage lineage cells. Precursor cells of polymorphonuclear leukocytes, such as myelocytes or metamyelocytes, make up a substantial amount of fresh bone marrow cells and greatly outnumber the precursors of macrophages (31). In the present study, we cultured bone marrow cells 1–2 days prior to PAF addition. Mature polymorphonuclear leukocytes were not expected to participate in this response, considering the life span of this type of cell. We have reported that PAF augmented glucose consumption of guinea pig peritoneal macrophage through binding itself to specific binding sites (12). Hartung reported that PAF stimulated oxidative burst in macrophages (32). However, no significant augmentation of candidacidal activity was observed with mineral oil-induced peritoneal macrophages, even when cells were incubated with PAF or its agonists. Peripheral cells may no longer be susceptible to PAF for inducing their microbicidal activity. The effector cells that mature during the cultivation could be precursor cells of macrophages. The suppressive effect on the induction of candidacidal activity by carrageenan or 2-chloroadenosine, reagents known as inhibitors against macrophages, further supports this idea. Natural killer cells present in bone marrow might be an alternative candidate for the responsible cell, because it has been recently reported that PAF modulated functions of natural killer cells (18). In our preliminary experiment, after 72 hr incubation with PAF, nonspecific esterase positive cells increased from 11% to 33%. The increased cell population could be monocytes and macrophages. Further study must be carried out to clarify the responsible cells.

So far, we do not know if PAF directly affects the induction of maturation of precursor cells into functionally mature cells. Production or secretion of some soluble factors, such as colony stimulating factors, would be triggered by PAF, and those factors affect the immature macrophages.

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REFERENCES

1. Benveniste, J., Henson, P.M., and Cochrane, C.G. (1972) *J. Exp. Med. 136,* 1356–1377.
2. Braquet, P., Touqui, L., Shen, T.Y., and Vargaftig, B.B. (1987) *Pharmacol. Rev. 39,* 97–145.
3. Cammusi, G., Bussolino, F., Tetta, C., Piacibello, W., and Aglietta, M. (1983) *Int. Arch. Allergy Appl. Immunol. 70,* 245–251.
4. Mencia-Huerta, J.M., and Benveniste, J. (1979) *Eur. J. Immunol. 9,* 409–415.
5. Lynch, J.M., Lotner, G.Z., Betz, S.J., and Henson, P.M. (1979) *J. Immunol. 123,* 1219–1226.
6. Lee, T.C., Lenihan, J., Malone, B., Roddy, L.L., and Wasserman, S.I. (1984) *J. Biol. Chem. 259,* 5526–5530.
7. Chignard, M., Le Couedic, J.P., Vargaftig, B.B., and Benveniste, J. (1980) *Br. J. Haematol. 46,* 455–464.
8. Camussi, G., Aglietta, M., Malavasi, F., Tetta, C., Piacibello, W., Sanavio, F., and Bussolino, F. (1983) *J. Immunol. 131,* 2397–2403.
9. Blank, M.L., Snyder, F., Byers, L.W., Brooks, B., and Muirhead, E.E. (1979) *Biochem. Biophys. Res. Commun. 90,* 1194–1200.
10. Shaw, J.O., Pinckard, R.N., Ferrigni, K.S., McManus, L.M., and Hanahan, D.J. (1981) *J. Immunol. 123,* 1847–1857.
11. Yasaka, T., Boxer, L.A., and Baehner, R.L. (1982) *J. Immunol. 128,* 1939–1944.
12. Hayashi, H., Kudo, I., Inoue, K., Onozaki, K., Tsushima, S., Nomura, H., and Nojima, S. (1985) *J. Biochem. 97,* 1737–1745.
13. Wardlaw, A.J., Moqbel, R., Cromwell, O., and Kay, A.B. (1986) *J. Clin. Invest. 78,* 1701–1706.
14. Findlay, S.R., Lichtenstein, L.M., Hanahan, D.J., and Pinckard, R.N. (1981) *Am. J. Physiol. 241,* C130–133.
15. Humphrey, D.M., McManus, L.M., Hanahan, D.J., and Pinckard, R.N. (1984) *Lab. Invest. 50,* 16–25.
16. Braquet, P., and Rola-Pleszczynski, M. (1987) *Immunol. Today 8,* 345–352.
17. Rola-Pleszczynski, M., Pignol, B., Pouliot, C., and Braquet, P. (1987) *Biochem. Biophys. Res. Commun. 142,* 754–760.
18. Rola-Pleszczynski, M., Pouliot, C., Pignol, B., and Braquet, P. (1987) *Prostaglandin 34,* 156.
19. Berdel, W.E., Bausert, W.R., Weltzien, H.U., Modolell, M.L., Widmann, K.H., and Munder, P.G. (1980) *Eur. J. Cancer 16,* 1199–1204.
20. Kudo, I., Nojima, S., Chang, H.W., Yanoshita, R., Hayashi, H., Kondo, E., Nomura, H., and Inoue, K. (1987) *Lipids 22,* 862–867.
21. Terashita, Z., Imura, Y., Takatani, M., Tsushima, S., and Nishikawa, K. (1987) *J. Pharmacol. Exp. Therapeu. 242,* 263–268.
22. Yoshida, K., Okamoto, M., Shimazaki, N., Hemmi, K. (1988) *Progr. Biochem. Pharmacol. 22,* 66–80.
23. Silvia-Hunter, M., and Cooper, B.H. (1974) in Manual of *Clinical Microbiology* (Lennette, E.H., Spauldig, E.H., and Truant, J.P., eds.) pp. 491–507, American Society for Microbiology, Washington, DC.
24. Nozawa, R.T., and Yokota, T. (1982) *Microbiol. Immunol. 26,* 153–161.
25. Allison, A.C., Harington, J.S., and Birbeck, M. (1966) *J. Exp. Med. 124,* 141–153.
26. Catanzaro, P.J., Schwartz, H.J., and Graham, R.C. (1971) *Am. J. Pathol. 64,* 387–399.

27. Tatsukawa, K., Mitsuyama, M., Takeya, K., and Nomoto, K. (1979) *J. Gen. Microbiol. 115*, 161–166.
28. Saito, T., and Yamaguchi, J. (1985) *J. Immunol. 134*, 1815–1822.
29. Yamashita, M., Homma, H., Inoue, K., and Nojima, S. (1983) *J. Toxicol. Sci. 8*, 177–188.
30. Honma, Y., Kasukabe, T., Hozumi, M., Tsushima, S., and Nomura, H. (1981) *Cancer Res. 41*, 3211–3216.
31. Clinical Hematology (1981) 8th edn. (Wintrobe, M.M., Lee, G.R., Boggs, D.R., Bithell, T.C., Foerster, J., Athens, J.W., and Lukens, J.N., eds.) p. 1890, Lea and Febiger, Philadelphia, PA.
32. Hartung, H.P. (1983) *FEBS Lett. 160*, 209–212.

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Lipid Composition of Normal Male Rat Islets

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Lipid composition was studied in fresh isolated islets from normal male rats. Extractable lipids represent 1856 μg per mg islet protein. In such extracts, phospholipids and neutral lipids represent 13.5% and 86.5%, respectively. Phosphatidylcholine (45.8%) and phosphatidylethanolamine (20.6%) were the major components of the phospholipid fraction, and phosphatidylinositol (8.9%) was the minor component. Esterified cholesterol (38.5%), cholesterol (25.5%) and free fatty acids (24.4%) were the major components of the neutral lipid fraction. Fatty acids esterified to phospholipids account for 619.7 pmol/islet, and 2710 pmol/islet were esterified to neutral lipids. In the phospholipid fraction, saturated and unsaturated fatty acids were in a similar proportion. Conversely, in the neutral lipids, two-thirds of the fatty acids were unsaturated. The ω6 family was the main component of the phospholipid unsaturated fatty acids. In the ω6 and ω3 families, the long-chain fatty acids represent the main components. In the neutral lipid fraction, a different percentage of each family was found: ω3 > ω6 > ω9. The long-chain polyunsaturated fatty acids were also predominant species in the ω6 and ω3 families. Further studies on the lipid composition of islets, obtained from rats with normal and altered islet functions, could provide new insights into the knowledge of the mechanism of insulin secretion.
Lipids 23, 1125–1128 (1988).

The ultrastructural and biochemical changes that occur in the pancreatic β-cell plasma membrane during emiocytosis, suggest that this organelle plays an active role in the last step of insulin secretion (1–3). However, several experimental evidences demonstrate that the cell membrane also participates in the earlier steps of this process, as a source of intracellular signals for the stimulus-secretion coupling (4). These signals would be released after the activation of several islet phospholipases (5). In this regard, the early release of arachidonic acid and its eicosanoid derivatives (6,8), diacylglycerol (9), inositol-1,4,5-triphosphate (IP_3) (10) and other phosphoinositides components (11), as well as phosphatidic acid (12), were reported by several authors. Moreover, the increment in the total islet-phospholipid turnover in response to several insulin secretagogue agents has also been demonstrated (4,13). All these data suggest that islet lipids, and particularly their phospholipid fractions, play an important role in the regulation of insulin secretion. Despite the large number of reports on this matter, little attention has been paid to the normal composition of islet lipids. This work was performed to provide such information through the quantitative determination of the different lipid fractions of fresh isolated islets from normal male rats.

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Abbreviations: (F)FA, (free) fatty acid; IP_3, inositol-1,4,5-triphosphate; NL, neutral lipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SPH, sphingomyelin; DBI, double bond index; (HP)TLC, (high performance) thin layer chromatography; R, DBI/saturated FA ratio.

MATERIAL AND METHODS

Adult normal male Wistar rats of ca. 200 g were fed ad libitum Purina Rat Chow (CABECA R N'3, Buenos Aires, Brazil). This chow contained 33% protein and 10% fat, and the following fatty acid composition: 16:0 (21.4%), 16:1 (2.1%), 18:0 (8.2%), 18:1 (24.9%), 18:2ω6 (37.7%) and 20:4ω6 (0.2%). The animals had free access to drinking water. In the morning of the experiment, nonfasted rats were killed by cervical dislocation without anaesthesia and the tail of the pancreas was carefully dissected. Pancreatic islets were isolated from the pancreas by collagenase (Serva Feinbiochemica, Heidelberg, Germany) digestion (14). After such digestion, separation of the islets from the acinar tissue was achieved by several washings with cold buffer, sedimentation and hand-picking under a stereomicroscope. Total lipids were extracted from groups of 150 islets with 19 ml of a mixture of chloroform/methanol (2:1, v/v) (15) and the washed lower phase was used for the analysis of the different lipid fractions. All solvents were of chromatographic grade. The total lipid content was determined by weighing the dry lipid extract resuspended in a small volume of chloroform. Neutral lipids (NL) were separated from phospholipids (PL) by thin layer chromatography (TLC) on plates of Silica Gel G-60, developed with chloroform/methanol/acetic acid/water (90:6:1.0:0.75, v/v/v/v) (16). The spots were identified by iodine staining, scraped and the NL were eluted with chloroform/methanol (2:1, v/v), and the Arvidson's method, chloroform/methanol/acetic acid/water (50:39:1:20, v/v/v/v) (17) was used for the elution of the PL. The extracts were evaporated to dryness under a stream of N_2. Recovery control was performed at every step using an islet-lipid extract labeled with [^{14}C]palmitic acid and appropriate standards labeled with [^{3}H]glycerol. Final values were adjusted to the corresponding percentage recovery. A double control was used to assess the percentage composition of islet PL and neutral lipids, i.e., a scanner measurement performed directly on the thin layer plates and the determination of radioactivity in the samples eluted from those plates. Identical percentage values were obtained using either method.

Different fractions of NL or PL were separated on HPTLC plates (Silica Gel 60 F254 precoated for HPTLC, E. Merck, Darmstadt, Federal Republic of Germany). A developing solvent mixture consisting of petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v) (18) was used to identify the different NL fractions. A solvent mixture of chloroform/methanol/30% aq. ammonia/water (140:50:7:3, v/v/v/v) and of chloroform/methanol/acetic acid/water (160:20:4:1.5, v/v/v/v) (19) was used to separate the PL fraction into its different constituents. Appropriate standards (P-L Biochemicals, Inc., Milwaukee, WI) were simultaneously run to identify each PL component. After chromatography, the lipids were charred for densitometry.

Plates were dipped into a 3%-cupric-acetate (w/v)-8%-phosphoric-acid (v/v) solution and heated at 180°C for 15 min (20). The plates were later scanned using a Shimadzu scanning densitometer in the reflectance mode.

Fatty acid (FA) methyl esters from PL or NL were obtained by refluxing the extracts for 3 hr at 65°C with HCl/methanol as described by Stöffel et al. (21). The methyl esters were then separated in a gas liquid chromatograph (GC-RIA Shimadzu) with a Supelco column (1-1851), 10% SP 2330 on 100/120 chromosorb AW Zog) and dual flame ionization detectors using a programmed temperature procedure.

The peaks obtained in the chromatograms were identified running appropriate standards of the purest available grade (P-L Biochemicals, Inc., Milwaukee, WI) and quantitatively estimated using the Δ11-20:1 FA (P-L Biochemicals, Inc., Milwaukee, WI) as internal standard.

Protein concentration was determined according to Lowry's method (22), using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as standard.

Statistical analysis of the data was performed using the Student's t-test for independent samples.

RESULTS

Fresh isolated rat islets comprised 1856 μg per mg of protein of total extractable lipids. Either the sum of the different islet-lipid fractions or the direct weight of the total islet-lipid extract gave similar figures for this parameter. From this total, the PL fraction represented 13.50%, and the remaining 86.5% corresponded to NL. Table 1 shows the different species identified in the PL islet fraction. The largest percentage corresponded to phosphatidylcholine (PC) (45.8%), followed by phosphatidylethanolamine (PE) (20.6%), phosphatidylserine (PS) (14%), sphingomyelin (SPH) (10.7%) and phosphatidylinositol (PI) (8.9%).

The different species identified in the islet-NL fraction are listed in Table 2. The major component was the esterified cholesterol (38.5%) followed by cholesterol and free fatty acids (FFA) (25.5% and 24.4%, respectively), and the triacylglycerol fraction, the minor component.

The different FA identified in the islet-PL NL fractions are presented in Table 3. The amount of FA esterified to PL was 619 ± 50.8 pmol/islet (n = 6), and 2710 ± 188 pmol/islet (n = 6) corresponds to the NL fraction.

TABLE 1

Phospholipid Composition of Fresh Isolated Islets

Phospholipids	ng/Islet	μg/mg Protein	%
Phosphatidylserine	22.9 ± 1.4	35.2	14.0
Phosphatidylinositol	14.4 ± 1.4	22.2	8.9
Sphingomyelin	17.5 ± 1.9	26.9	10.7
Phosphatidylcholine	74.6 ± 5.2	114.8	45.8
Phosphatidylethanolamine	33.5 ± 4.1	51.5	20.6
Total	162.9 ± 11.10	250.6	

Phospholipid fractions were separated by HPTLC and the different species identified and quantitatively measured, as described in Material and Methods. Each value represents the mean ± SEM of four different islet homogenates.

A similar percentage of saturated and unsaturated FA was found in the phospholipid fraction. Conversely, in the NL fraction, two-thirds of the FA were unsaturated. Accordingly, the double bond index (DBI) and the DBI/saturated FA ratio (R) were larger in the NL than in the PL fraction.

The ω6 family represents the main component of the PL-unsaturated FA. In this family, the largest components were those with 20 and 22 carbons. A similar composition has been described in the outer acrosomal membranes of mammalian sperm (23). The long-chain polyunsaturated fatty acids also represent the main components of the ω3 family. A different percentage of each family was found in the NL fraction, namely ω3 > ω6 > ω9. As

TABLE 2

Neutral Lipid Composition of Fresh Isolated Islets

Lipid	ng/Islet	μg/mg Protein	%
Cholesterol	266 ± 22	409	25.5
Free fatty acids	254 ± 10	391	24.4
Triacylglycerol	122 ± 12	188	11.7
Esterified cholesterol	401 ± 13	618	38.5
Total	1043 ± 29	1606	

Lipids were fractioned and quantitatively measured as described in the text. Each value represents the mean ± SEM of four different islet homogenates.

TABLE 3

Fresh Islet Fatty Acids Composition

Fatty acid	Phospholipis	Neutral lipids
12:0	0.8 ± 0.2	0.8 ± 0.1
14:0	3.3 ± 0.5	3.3 ± 0.2
15:0	1.5 ± 0.3	2.7 ± 0.4
16:0	22.5 ± 2.6	14.4 ± 1.0
16:1	6.1 ± 0.9	5.0 ± 0.5
18:0	15.1 ± 1.7	7.1 ± 0.7
18:1ω9	13.3 ± 1.3	7.2 ± 0.4
20:1ω9	0.2 ± 0.0	1.5 ± 0.3
20:3ω9	0.3 ± 0.1	0.6 ± 0.2
18:2ω6	4.2 ± 0.4	1.9 ± 0.1
18:3ω6	0.7 ± 0.3	1.0 ± 0.1
20:2ω6	0.1 ± 0.0	1.0 ± 0.2
20:3ω6	0.7 ± 0.3	1.8 ± 0.1
20:4ω6	4.6 ± 0.6	3.9 ± 0.3
22:2ω6	5.6 ± 1.1	4.0 ± 0.9
22:4ω6	4.5 ± 0.7	5.4 ± 0.5
22:3ω6	3.2 ± 0.7	4.3 ± 0.6
18:3ω3	1.3 ± 0.2	2.4 ± 0.3
20:5ω3	0.3 ± 0.2	3.4 ± 0.3
22:3ω3	4.8 ± 0.9	7.9 ± 0.9
22:4ω3	3.2 ± 0.7	5.0 ± 0.6
22:5ω3	2.9 ± 1.1	7.0 ± 1.2
22:6ω3	0.7 ± 0.5	8.4 ± 1.2
Sat FA	43.3	28.5
DBI	141.7	240.8
R	3.3	8.5

The lipids were interesterified and the resulting fatty acid methyl esters were analyzed as described in the text. Each value represents the mean percentage ± SEM of four determinations; DBI = double bond index; R = DBI/saturated FA ratio.

in the PL fraction, long-chain polyunsaturated FA were the predominant species found in the ω6 and ω3 families.

DISCUSSION

It is well known that the lipid composition of animal tissues, even among a given species, varies from one tissue to another, being affected by several factors such as diet composition, sex, age, circadian rhythms and environmental conditions (24). In our experimental model, the effect of the enzymes from the exocrine pancreas released during the isolation of islets, could also represent another source of variation. In islets isolated from normal nonfasted male Wistar rats, such composition shows an uneven proportion of polar (13.5%) and neutral lipids (86.5%).

The total content of PL currently described, expressed on a per-islet basis, agrees with the values reported by Hallberg (25) in Wistar rats. Conversely, they are significantly lower than the values reported by Montague and Parkin (26) in guinea pig islets. According to Hallberg (25), this difference probably reflects species variations of the total PL content of islets. Hallberg stated also that the total content of PL is not affected by different conditions like 48-hr starvation, the culture of the isolated islets for one week, the addition of leucine and gliclazide to or the removal of Ca^{2+} from such medium (25). We have previously shown that the PL content was significantly lower in islets isolated from hypothyroid rats (27). Hence, those results might indicate that chronic rather than acute conditions (or effects) can modify the total content of islet PL, which could affect the release of insulin.

PL fractions identified in the islets are similar to those described in other rat tissues. PC and PE are their major components, as in normal liver (24,28) with PS instead of PI being the minor component in the islets. Otherwise, the percentages of PC and PE are lower in the islets, but PS and SPH are higher than in the liver. The relative content of the different PL classes currently described in the islets isolated from normal nonfasted Wistar rats, with PC and PE as major components, coincides with the data reported from obese hyperglycemic (29) or NMRI mice (25) and Sprague-Dawley rats (30). This islet PL composition is significantly altered in some pathological conditions such as hypothyroidism (27), which is accompanied by a decrease in the insulin secretory response to glucose. These data suggest that the normal islet-PL composition might be a condition necessary to achieving an adequate β-cell secretory response.

The amount of NL per mg of protein was similar in liver and isolated islets (31). However, the esterified cholesterol, the cholesterol and the FFA fractions were larger, but the triacylglycerol fraction was lower in the islets compared to liver.

The islets, like other rat tissues, present a complex composition of esterified FA, with a clear predominance of even-numbered acids (32). Berne has concluded that the major source for these FA is their extracellular uptake rather than their biosynthesis (33). Palmitic acid was the largest component of the saturated FA as in the heart (34). This fact could, at least in part, explain: a) the high and concentration-dependent oxidation rate of palmitic acid measured in islets (35) and b) the large incorporation of labeled palmitate into the islet lipids by islets incubated in the presence of different insulin secretagogue agents (36).

Turk et al. have already reported on the FA composition of the major glycerolipids from Sprague-Dawley-rat islets cultured overnight and then incubated with glucose (30). No references are given in this paper on the FA composition in freshly untreated islets. As shown in our Table 3, they also found that palmitate and stearate were the predominant saturated FA esterified to PL. Conversely, some discrepancies were found in the composition of unsaturated FA. We have previously reported that the percentage composition of polyunsaturated FA changed significantly in islets incubated with high glucose (37). This effect was attributed to a possible effect of glucose on the activity of islet desaturases and the FA chain-elongation process (37). Hence, the different metabolic conditions of the islets used in the current report and those mentioned above could, at least in part, explain the apparent discrepancies in the percentage distribution of polyunsaturated FA. The different number of FA considered in Turk's study compared with our own (11 vs 22), could also contribute to such a difference. However, we do not have a plausible explanation for the particular lower percentage of arachidonic acid found in our experiments.

A large number of a long-chain polyunsaturated FA was found in the islets, mainly esterified to the neutral lipids. The richness of polyunsaturated FA is a characteristic of the lipid composition found in very active tissues, such as heart and liver (38). Such composition explains the large DBI and R (DBI/saturated acid ratio) found in the islets (37). The degree of unsaturation of the phospholipid acyl chains has been considered one of the main determinants of the microviscosity of lipid regions (39). Assuming that the PL content grossly represents the islet membrane composition, the values obtained for such a parameter would suggest that, in the islets, the microviscosity of the membrane lipids is lower than in the liver membranes.

It has already been reported that the oxidation of endogenous FA accounts for about 30% of islet basal respiratory rate (40). The large amount of endogenous islet FA, mainly esterified to the neutral lipid fraction, could easily provide such an oxidizable substrate. In addition, the FA also represent the source of intracellular signals for the stimulus-secretion coupling. The change in the mass of FA esterified to PC (30), the release of arachidonic acid (6,7) and its eicosanoid derivatives (6-8,36) under the glucose stimulus are clear examples of such function.

Further, more detailed studies on the composition and metabolism of islet lipids in normal and pathological conditions would provide important evidence for a more comprehensive knowledge of the insulin secretion mechanism.

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REFERENCES

1. Orci, L. (1982) *Diabetes 31,* 538-565.
2. Lacy, P.E. (1961) *Am. J. Med. 31,* 851-859.
3. Semino, M.C., Gagliardino, E.P., and Gagliardino, J.J. (1987) *Acta Physiol. Pharmacol. Latinoam. 37,* 533-539.
4. Best, L., and Malaisse, W.J. (1983) *Diabetologia 25,* 299-305.
5. Kelly, K.L., and Laychock, S.G. (1981) *Prostaglandins 21,* 759-769.
6. Gagliardino, J.J., Borelli, M.I., Gagliardino, E.P., and García, M.E. (1986) *Diabetes Res. Clin. Practice 1,* 327-333.
7. Wolf, B.A., Turk, J., Sherman, W.R., and McDaniel, M.L. (1986) *J. Biol. Chem. 261,* 3501-3509.
8. Robertson, R.P. (1988) *Diabetes 37,* 367-370.
9. Tanigawa, L., Kuzuya, H., Imura, H., Taniguchi, H., Baba, S., Takai, Y., and Nishizyku, Y. (1982) *FEBS Lett. 138,* 183-186.
10. Prentki, M., Biden, T.J., Janjic, D., Irvine, R.F., Berridge, M.J. and Wollheim, C.B. (1984) *Nature 309,* 562-564.
11. Zawalich, W.S. (1988) *Diabetes 37,* 137-141.
12. Dunlop, M.E., Larkins, R.G., and Court, J.M. (1982) *FEBS Lett. 144,* 259-263.
13. Best, L., Dunlop, M., and Malaisse, W.J. (1984) *Experientia 40,* 1085-1091.
14. Lacy, P.E., and Kostianovsky, M. (1967) *Diabetes 16,* 35-39.
15. Folch, J., Lee, S.M., and Sloan-Stanley, G.M. (1957) *J. Biol. Chem. 226,* 497-509.
16. Van Dorp, D.A., Beerthuis, R.K., Nugteren, D.W., and Vankerhan, H. Jr. (1964) *Biochim. Biophys. Acta 10,* 204-206.
17. Arvidson, G.A.E. (1968) *Eur. J. Biochem. 4,* 478-486.
18. Malins, D., and Mangold, H.K. (1960) *J. Am. Oil Chem. Soc. 37,* 576-578.
19. Neskovic, N.M., and Kostic, D.M. (1968) *J. Chromatogr. 35,* 297-300.
20. Macala, L.J., Yu, R.K., and Ando, S. (1983) *J. Lipid, Res. 24,* 1243-1250.
21. Stöffel, O.W., Chu, F., and Ahrens, E.W., Jr. (1959) *Anal. Chem. 31,* 307-308.
22. Lowry, O.W., Rosebrough, N.J., Farr, A.L., and Randall, R.H. (1951) *J. Biol. Chem. 193,* 265-275.
23. Nikolopoulou, M., Soucek, D.A., and Vary, J.C. (1986) *Lipids 21,* 566-570.
24. Ruggieri, S., and Fallani, A. (1979) *Lipids 14,* 323-333.
25. Hallberg, A. (1984) *Biochim. Biophys. Acta 796,* 328-335.
26. Montague, W., and Parkin, E.N. (1980) *Horm. Metab. Res. (Suppl) 10,* 153-157.
27. Cortizo, A.M., García, M.E., Pasquini, J.M., and Gagliardino, J.J. (1984) *IRCS Med. Sci. 12,* 1117-1118.
28. Upreti, G.C., de Antueno, R.J., and Wood, R. (1983) *J. Natl. Cancer Inst. 70,* 567-573.
29. Fex, G., and Lernmarck, A. (1972) *FEBS Lett. 25,* 287-291.
30. Turk, J., Wolf, B.A., Lefkowith, J.B., Stump, W.T., and McDaniel, M.L. (1986) *Biochim. Biophys. Acta 879,* 399-409.
31. Upreti, G.C., de Antueno, R.J., and Wood, R. (1983) *J. Natl. Cancer Inst. 70,* 559-566.
32. Brenner, R.R., Mercuri, O., and De Tomas, M.E. (1962) *J. Nutr. 77,* 203-209.
33. Berne, C. (1975) *Biochem. J. 152,* 667-673.
34. Peluffo, R.O., and Brenner, R.R. (1962) *An. Asoc. Quím. Argent. 50,* 1-12.
35. Berne, C. (1975) *Biochem. J. 152,* 661-666.
36. Laychock, S.G. (1983) *Diabetes 32,* 6-13.
37. Cortizo, A.M., García, M.E., and Gagliardino, J.J. (1987) *Med. Sci. Res. 15,* 289-290.
38. Di Corleto, P.E., Warrach, J.B., and Silversmith, D.B. (1979) *J. Biol. Chem. 254,* 7795-7802.
39. Shinitzky, M., and Inbar, M. (1976) *Biochim. Biophys. Acta 433,* 133-149.
40. Malaisse, W.J., Malaisse-Lagae, F., Sener, A., and Hellerstrom, C. (1985) *Biochem. J. 227,* 995-1002.

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New Oxidation Products of 2,2,5,7,8-Pentamethyl-6-chromanol

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Oxidation of the α-tocopherol model compound 2,2,5,7,8-pentamethyl-6-chromanol (*1*) by *t*-butyl hydroperoxide in chloroform, to which an alcohol has been added, produces 5-alkoxymethyl-2,2,7,8-tetramethyl-6-chromanol as the major product. In the present study, *1* was oxidized by *t*-butyl hydroperoxide in water-saturated chloroform to determine whether water would influence product formation in the same way as alcohols.

In addition to the usual products of oxidation such as 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (*9*), 5-formyl-2,2,7,8-tetramethyl-6-chromanol (*11*), the spirodimer and spirotrimer of *1*, three new products have been identified—2,2,7,8-tetramethyl-5-(2,2,5,7,8-pentamethyl-6-chromanoxy)methyl-6-chromanol (*4*), 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (*5*) and 3-hydroxymethyl-2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-1,4-benzoquinone (*7*).
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Oxidation of α-tocopherol by *t*-butyl hydroperoxide in purified chloroform, to which ethanol has been added, produces 5-ethoxymethyl-7,8-dimethyltocol in amounts which vary with the concentration of ethanol (1). In the absence of ethanol, oxidation does not occur.

More recent work has shown that, when the α-tocopherol model compound 2,2,5,7,8-pentamethyl-6-chromanol (1) is oxidized by *t*-butyl hydroperoxide in chloroform, to which various alcohols have been added, the predominant product in all cases, in fair to good yield, is the 5-alkoxymethyl-2,2,7,8-tetramethyl-6-chromanol (C. Suarna and P. T. Southwell-Keely, unpublished data). The 5-alkoxymethyl derivatives are formed by oxidation of the chroman to the quinone methide to which the alcohol is then added. Because this was a very facile reaction, it was decided to examine whether water would behave in the same way as an alcohol.

MATERIALS AND METHODS

IR spectra were determined on a Perkin Elmer 580B spectrometer, UV spectra on a Perkin Elmer 124 double beam spectrophotometer and ^1H and ^{13}C NMR spectra on a Bruker AM 500 spectrometer. NMR spectra were taken in $CDCl_3$ and are reported in parts per million downfield from tetramethylsilane as internal standard. Electron impact mass spectra were determined at 70 ev on an A.E. I MS12 spectrometer. High resolution molecular weights were determined on a Bruker Spectrospin Fourier Transform Ion Cyclotron Resonance spectrometer.

Chloroform was purified by washing with 18 M sulfuric acid, distilled water until the washings were neutral, drying (Na_2SO_4) and distilling immediately before use.

t-Butyl hydroperoxide (70%, EGA CHEMIE, Steinheim, West Germany) was purified by the sodium salt method (2). Purity (iodometrically) was 95%.

1 together with its spirodimer and spirotrimer, 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (5), 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (9) and 5-formyl-2,2,7,8-tetramethyl-6-chromanol (11) were prepared as reference compounds by known methods (3–8).

Oxidation of 1 by t-butyl hydroperoxide in the presence of water. To 1 (200 mg, 0.91 mmol) in chloroform (30 ml, saturated with water) was added *t*-butyl hydroperoxide (82 mg, 0.91 mmol) and the solution was heated under reflux for 3 hr at 60°C. The solution was washed with 5% ferrous sulfate (3 × 20 ml), water (3 × 20 ml), dried (Na_2SO_4) and the solvent removed in vacuo. The residue was chromatographed on thin layers of Silica Gel GF_{254} (solvent: light petroleum (bp 60–80°C)/ethyl acetate (9:1)), the spots were located under ultraviolet light, eluted with purified chloroform, and the solvent was removed under a stream of nitrogen and weighed. Major bands with the following R_f's were isolated: 0.00 (5.5 mg), 0.06 (15 mg), 0.09 (0.9 mg), 0.23 (1.6 mg), 0.30 (2.4 mg), 0.37 (3.1 mg), 0.42 (128 mg; 1), 0.47 (2.3 mg), 0.49 (1.6 mg; 11), 0.52 (8.5 mg; spirodimer of 1) and 0.55 (16.4 mg; spirodimer of 1 plus unknown).

Major band R_f 0.00 was rechromatographed on Silica Gel GF_{254} (solvent: light petroleum (bp 60–80°C)/ethyl acetate (8:2)/benzene (9:1)) yielding 3 minor bands with R_f's 0.03, 0.15 and 0.24.

Minor band R_f 0.15 (4.7 mg) was identified as (7a*S*,11a*R*)-1*H*-2,3-dihydro-7a-(3-hydroxy-3-methylbutyl)-3,3,5,6,9,10,11a-heptamethylpyrano [2,3-a]xanthene-8,11(7a*H*,11a*H*)-dione (12) (9) and minor band R_f 0.24 (0.8 mg) was identified as 9.

Minor band R_f 0.03 (0.5 mg) was identified as 3-hydroxymethyl-2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-1,4-benzoquinone (7) and had UV λ_{max} 260 nm; IR (KBr) 3422 (OH), 2972, 2929, 2858, 1704, 1649 (C=C-C=O), 1458, 1383, 1306, 1279, 1223, 1150, 1127, 1021, 937, 912, 732 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.28 (s, 6 H, 2 × CH_3), 1.59 (m, 2 H, =C-CH_2-CH_2-), 2.027 (s, 3 H, =C-CH_3), 2.031 (s, 3 H, =C-CH_3), 2.66 (m, 2 H, =C-CH_2-), 4.57 (s, 2 H, =C-CH_2-OH); irradiation of the singlet at 4.57 ppm gave a Nuclear Overhauser Enhancement (NOE) on the multiplet at 2.66 ppm, thus proving that the hydroxymethyl and the methylene groups were attached to adjacent carbon atoms of the quinone ring; ^{13}C NMR ($CDCl_3$) δ 12.17, 12.47, 21.09, 29.24, 29.24, 43.24, 57.27, 71.05, 140.08, 140.71, 141.36, 145.96, 187.58, 188.59. MS (ei) *m/z* (rel. intensity) 252 $[M]^+$ (6), 234 $[M^+ - H_2O]$ (33), 221 $[M^+ - CH_2 - OH]$ (34), 216 $[M^+ - 2 \times H_2O]$ (29), 201 (44), 191 (35), 176 (100). Exact mass (self ci) calc. for $C_{14}H_{21}O_4$ 253.143436; Found 253.1496650.

Major bands R_f's 0.06 and 0.09 were combined and rechromatographed on Silica Gel GF_{254} (solvent: light petroleum (bp 60–80°C)/ethyl acetate (8:2)/benzene (9:1)) to yield minor bands with R_f's 0.03 (7), 0.22 (9; 10 mg), 0.34 (see below), 0.39 (unknown), 0.45 (unknown), 0.60 (1), 0.69 (11), 0.72 (unknown), 0.76 (unknown).

Minor band R_f 0.34 (3.7 mg), identified as 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (5), was identical with the synthetic material and had UV λ_{max} 300 nm; IR

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Abbreviation: NOE, Nuclear Overhauser Enhancement.

(KBr) 3387 (OH), 2978, 2932, 2861, 1685, 1639, 1624, 1585, 1455, 1386, 1372, 1273, 1230, 1171, 1127, 1093 (C-O-C), 976, 929, 902, 852, 699, 656, 603 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.28 (s, 6 H, 2 × CH_3), 1.77 (t, 2 H, 3-CH_2, J = 6.9 Hz), 1.98 (t, 1 H, -CH_2-*OH*, J = 5.66 Hz), 2.11 (s, 3 H, Ar-CH_3), 2.16 (s, 3 H, Ar-CH_3), 2.65 (t, 2 H, 4-CH_2, J = 6.9 Hz), 4.875 (d, 2 H, Ar-*CH_2*-OH, J = 5.5 Hz), 7.03 (s, 1 H, -OH); irradiation of the doublet at 4.875 ppm gave an NOE on the singlet at 7.03 ppm and on the triplet at 2.65 ppm, thus confirming that the hydroxymethyl group was on the 5-position of the chroman ring; ^{13}C NMR ($CDCl_3$) δ 11.77, 11.99, 20.18, 26.70, 26.70, 32.90, 60.07, 72.57, 114.99, 118.85, 123.08, 125.83, 145.10, 147.34. MS (ei) *m/z* (rel. intensity) 236 $[M]^+$ (34), 218 $[M^+ - H_2O]$ (95), 203 (84), 189 (28), 175 (100). Exact mass calc. for $C_{14}H_{20}O_3$ 236.140696; Found 236.1422894.

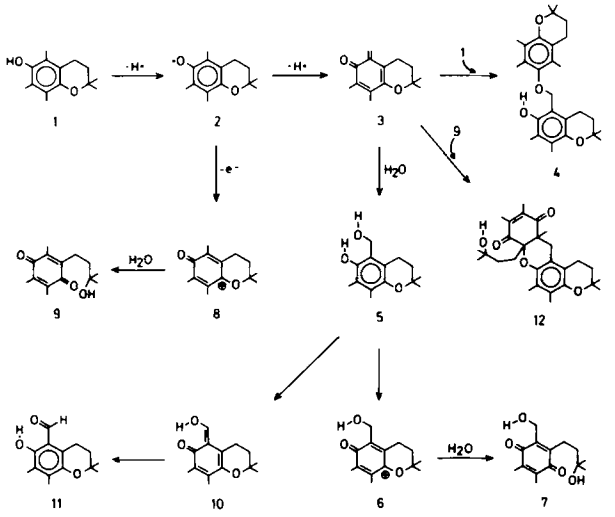
Major band R_f 0.55 was rechromatographed on Silica Gel GF_{254} (solvent: light petroleum (bp 60–80°C)/ethyl acetate (8:2)/benzene (0.5:9.5)) to yield minor bands with R_f 0.00 (**9**), 0.36 (spirodimer of **1**), 0.50 (spirotrimer of **1**; 8 mg), 0.61 (see below) and 0.72 (unknown).

Minor band R_f 0.61 (3 mg) was identified as 2,2,7,8-tetramethyl-5-(2,2,5,7,8-pentamethyl-6-chromanoxy)-methyl-6-chromanol (**4**) and had UV λ_{max} 291 nm; IR (KBr) 3408 (OH), 2978, 2932, 2874, 1457, 1385, 1371, 1273, 1228, 1171, 1128, 1091 (C-O-C), 974, 928, 902, 855, 760 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.28 (s, 6 H, 2 × CH_3), 1.31 (s, 6 H, 2 × CH_3), 1.74 (t, 2 H, 3-CH_2, J = 6.9 Hz), 1.80 (t, 2 H, 3′-CH_2, J = 6.9 Hz), 2.10 (s, 3 H, Ar-CH_3), 2.13 (s, 3 H, Ar-CH_3), 2.18 (s, 3 H, Ar-CH_3), 2.20 (s, 3 H, Ar-CH_3), 2.22 (s, 3 H, Ar-CH_3), 2.50 (t, 2 H, 4-CH_2, J = 6.9 Hz), 2.61 (t, 2 H, 4′-CH_2, J = 6.9 Hz), 4.92 (s, 2 H, Ar-*CH_2*-O-), 7.8 (s, 1 H, -OH); irradiation of the singlet at 4.92 ppm gave an NOE on the singlet at 7.8 ppm and on the triplet at 2.50 ppm, thus proving that the second chroman was attached at the 5-position of the first chroman ring; ^{13}C NMR ($CDCl_3$) δ 11.92, 12.00, 12.00, 12.27, 13.13, 20.17, 21.05, 26.68, 26.68, 26.82, 26.82, 29.74, 32.87, 71.77, 72.44, 73.01, 114.65, 116.20, 117.69, 123.39, 123.59, 125.76, 125.89, 127.78, 144.95, 147.41, 147.79, 148.65. MS (ei) *m/z* (rel. intensity) 438 $[M]^+$ (45), 421 (6), 232 (6), 220 (74), 219 (100), 218 (17), 203 (13). Exact mass calc. for $C_{28}H_{38}O_4$ 438.276461; Found 438.2778098.

RESULTS AND DISCUSSION

Oxidation of **1** occurs in two single electron steps leading first to the chromanoxyl radical (**2**) (10–13), and then to the quinone methide (**3**) or phenoxylium species (**8**) (Scheme 1) (14,15). Neither **3** nor **8** is stable and both react further either by nucleophilic addition or, in the case of **3**, by polymerization.

Addition of water to **8** produces **9**, the first recognized oxidation product of **1** (7). It would be expected that water would also add to **3** to produce **5** (Scheme 1). This compound, although known as a product of synthesis (6), does not appear to have been reported as an oxidation product. The yield of **5** is much less than that of 5-alkoxymethyl-2,2,7,8-tetramethyl-6-chromanols formed by addition of alcohols to **3** under similar conditions (C. Suarna and P. T. Southwell-Keely, unpublished data). There are probably several reasons for this. First, the much greater solubility in chloroform of alcohols than of water would produce



SCHEME 1

a homogeneous rather than a heterogeneous reaction and lead to a much higher concentration of alcohol in the reaction. Second, **5** is an unstable product (see below) and reacts further, but the 5-alkoxymethyl-2,2,7,8-tetramethyl-6-chromanols are relatively stable.

7 could be formed by two routes. The first route involves oxidation of **5** to the corresponding phenoxylium species **6** followed by addition of water to form the hemiketal and ring opening to the quinone **7** (Scheme 1). The second route (not shown) involves the enolization of **9** to a quinone methide (16), addition of water to form a hydroxymethyl hydroquinone followed by oxidation to the hydroxymethyl quinone. The first route is considered the more likely because **5**, on exposure to air at room temperature, oxidizes to a mixture of **7** and **11**, plus several minor unidentified products (C. Suarna and P. T. Southwell-Keely, unpublished data). By contrast, **9** was completely stable to oxidation by *t*-butyl hydroperoxide at 60°C for 6 hr in water-saturated chloroform.

It is worth noting that when **5** oxidizes spontaneously to a mixture of **7** and **11**, **11** is the major product. The reason for this may be that, although **10** and **6** are of equivalent oxidation state, **10** is merely the enol of **11**, into which it readily converts, but formation of **7** from **6** requires additional reaction with water.

The identification of **4** brings to four—the number of characterized dimers from the oxidation of **1**. The mode of formation of each of these dimers is different. **4** is undoubtedly formed by the nucleophilic addition of a second molecule of **1** to **3** and is a logical expectation, given the ease with which a variety of alcohols adds to **3** to form 5-alkoxymethyl derivatives (1; C. Suarna and P. T. Southwell-Keely, unpublished data). The dihydroxy dimer is formed by free-radical dimerization (4). The spirodimer is formed by Diels-Alder cycloaddition involving two molecules of **3**, one of which acts as diene and the second as dienophile (4,17), and **12** is formed by Diels-Alder cycloaddition of one molecule of **3** to one molecule of **9** (9).

REFERENCES

1. Sumarno, M., Atkinson, E., Suarna, C., Saunders, J.K., Cole, E.R., and Southwell-Keely, P.T. (1987) *Biochim. Biophys. Acta 920,* 247–250.

2. Barnard, D., and Hargrave, K.R. (1951) *Anal. Chim. Acta 5,* 476-488.
3. Smith, L.I., Ungnade, H.E., Hoehn, H., and Wawzonek, S. (1939) *J. Org. Chem. 4,* 311-317.
4. Skinner, W.A., and Alaupovic, P. (1963) *J. Org. Chem. 28,* 2854-2858.
5. Skinner, W.A., and Parkhurst, R.M. (1964) *J. Org. Chem. 29,* 3601-3603.
6. Nakamura, T., and Kijima, S. (1972) *Chem. Pharm. Bull. 20,* 1681-1686.
7. John, W., Dietzel, E., and Emte, W. (1939) *Hoppe-Seyler's Z. Physiol. Chem. 257,* 173-189.
8. Fujimaki, M., Kanamaru, K., Kurata, T., and Igarashi, O. (1970) *Ag. Biol. Chem. 34,* 1781-1787.
9. Suarna, C., Craig, D.C., Cross, K.J., and Southwell-Keely, P.T. (1988) *J. Org. Chem. 53,*1281-1284.
10. Mukai, K., Tsuzuki, N., Ishizu, K., Ouchi, S., and Fukuzawa, K. (1981) *Chem. Phys. Lipids 29,* 129-135.
11. Mukai, K., Tsuzuki, N., Ouchi, S., and Fukuzawa, K. (1982) *Chem. Phys. Lipids 30,* 337-345.
12. Matsuo, M., Matsumoto, S., and Ozawa, T. (1983) *Org. Mag. Res. 21,* 261-264.
13. Doba, T., Burton, G.W., and Ingold, K.U. (1983) *J. Am. Chem. Soc. 105,* 6505-6506.
14. Skinner, W.A., and Parkhurst, R.M. (1966) *J. Org. Chem. 31,* 1248-1251.
15. Nilsson, J.L.G., Branstad, J.O., and Sievertsson, H. (1968) *Acta Pharm. Suec. 5,* 509-516.
16. Turner, A.B. (1964) *Q. Rev. Chem. Soc. 28,* 347-360.
17. Nelan, D.R., and Robeson, C.D. (1962) *J. Am. Chem. Soc. 84,* 2963-2965.

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Molecular Species of Mycolic Acid Subclasses in Eight Strains of *Mycobacterium smegmatis*

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Thin layer chromatographic and gas chromatographic separation and mass spectrometric identification of mycolic acid subclasses and molecular species from eight strains of *Mycobacterium smegmatis* were established. Two major adjacent spots and a lower minor one were detected on silica gel thin layer chromatograms of methyl esters. The most abundant subclass showing the highest R_f value on TLC was that of α-mycolic acids (M_1), the second was that of α′-mycolic acids (M_1'), a shorter homologue than α-mycolates, and the third was the hydroxy mycolic acids (M_4) derived from epoxy mycolic acids. They were identified by gas chromatography–mass spectrometry as their trimethylsilylether derivatives. α′-Mycolic acids were monoenoic acids ranging from C_{60} to C_{66} and possessing an α-unit of $C_{24:0}$. Such profiles of α′-mycolic acids were common in eight strains. α-Mycolates were dienoic acids ranging from C_{75} to C_{79} and possessing an α-unit of $C_{24:0}$. In most strains, the major molecular species of α-mycolates were odd-carbon-numbered, centering at C_{77} and C_{79}, possessing a methyl branch in the even-carbon-numbered straight chain. The average carbon number of α-mycolates, from seven strains examined, was about 78, but that of the Takeo strain was 76.3. The profiles of epoxy mycolic acid molecular species composition from eight strains ranging from C_{75} to C_{81} were very similar to their M_1 subclass profiles.
***Lipids 23,* 1132–1138 (1988).**

Mycolic acids, high-molecular weight β-hydroxy, α-branched fatty acids, are the most characteristic components found in the cell wall lipids of mycobacteria (1–7) and related groups such as nocardiae (8–10), rhodococci (11,12) and corynebacteria (13–15). They differ from species to species in their structure and composition. The structures of mycolic acids contribute to the physicochemical properties of the cell walls in these bacteria (16–19). Nocardiae, corynebacteria and rhodococci have one subclass of mycolic acids (α-mycolic acids) that can be separated by thin layer chromatography (TLC). On the other hand, the composition of mycobacterial mycolic acids is much more complex and specific to each species. In recent years, we have developed an analytical method for the determination of individual molecular species of mycolic acids using gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) of trimethylsilylated (TMS) derivatives (12,20–26). Analysis of the mycolic acid composition appears to be very useful for the chemotaxonomy of mycobacteria.

Mycobacterium smegmatis, a species of rapidly growing mycobacteria, is widely distributed in nature. This species has been most frequently used as the source for studies on mycobacterial metabolism and enzyme systems (27–30). However, Tsukamura and coworkers reported that *M. smegmatis* was taxonomically heterogenous (31). Moreover, there are only a few reports about the mycolic acids of *M. smegmatis* (4,6,32–36). The present paper describes the detailed analysis of mycolic acid molecular species in eight strains of *M. smegmatis* using our GC–MS method.

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Abbreviations: TMS, trimethylsilyl; GC, gas chromatography; MS, mass spectrometry; TLC, thin layer chromatography.

MATERIALS AND METHODS

The *M. smegmatis* strains used in the present work were Takeo, Nishino, PM-5, SN-2, Jucho, Butyricum, Rabinowitch and Sekiguchi. The latter seven strains were donated by Dr. Tokunaga, Department of Tuberculosis, National Institute of Health, Tokyo. Cells were grown in a medium containing 1% glucose, 1% peptone and 0.5% yeast extract, with the pH adjusted to 7.4, on a rotary shaker at 37°C. After 6 days, the cells were harvested by centrifugation and washed with distilled water. The packed cells were suspended in a solution of 10% KOH in 80% aqueous methanol and heated under reflux for 5 hr. After acidification of the aqueous phase, the total fatty acids were extracted with hexane and converted to the methyl esters by refluxing with benzene/methanol/H_2SO_4 (10:20:1, v/v/v) for 2 hr, taking care because benzene is a potent carcinogen. The methyl esters of mycolic acids were separated into three subclasses on a thin layer plate of Silica Gel G (Analtec Inc., Newark, DE) with a solvent system of hexane/diethyl ether (4:1, v/v). The subclasses were then visualized with iodine vapor and recovered from the gel with chloroform. The methyl esters of mycolic acids were trimethylsilylated with N,O-bistrimethylsilyltrifluoroacetamide/pyridine (2:1, v/v) at 80°C for 30 min, as described earlier (12,22). Hydrogenation of mycolic acid methyl esters was performed with PtO_2 in chloroform with or without acetic acid for 3 hr at room temperature. Gas chromatography of the trimethylsilylated derivatives of mycolic acid methyl esters was carried out with a Shimazu GC-6A apparatus equipped with a hydrogen flame ionization detector. GC–MS was performed on a Hitachi M-80B Double Focusing apparatus. The glass column (0.4 m × 3 mm) was packed with 2% OV-101, and maintained isothermally at 315°C or 330°C. The injection port and the flame ionization detector were maintained at 390°C. The molecular separator was kept at 365°C. The ion source energy was 20 eV, and the accelerating voltage was 3 kV. The mycolic acid composition of each subclass was determined by gas chromatography or mass chromatography peaks. The mass spectra of mycolic acid esters were recorded repeatedly at appropriate intervals, the data were compiled in a data processing system (Hitachi M-0101), and then the bar graphs were reproduced as mass spectra of each top component of the total ion current peak or as the average values of several scans. The mass chromatograms

were obtained by continuously monitoring the mass number of each $[M-15]^+$ ion produced from the molecular species of TMS methyl mycolate possessing certain carbon and double bond numbers and those of $[B-29]^+$ ions derived from the α-units.

RESULTS

Figure 1 shows the thin layer chromatographic profiles of fatty acid methyl esters from eight strains of *M. smegmatis*. The total fatty acid methyl esters from each strain gave three major and one minor spots. The upper spot corresponded to nonpolar fatty acid esters. The lower spots, designated as M_1, M_1' and M_4, corresponded to α-, α'- and hydroxy (or hydroxymethoxy) mycolic acid esters in their mobilities, respectively (35–37). Each subclass of these mycolic acid esters was converted to its TMS derivative and analyzed by gas chromatography.

The profiles of gas chromatograms of TMS derivatives of M_1' from eight strains of *M. smegmatis* were very similar to each other and showed four major peaks corresponding to C_{60}, C_{62}, C_{64} and C_{66} acid esters, as judged from the relationship between log retention time vs carbon numbers (Fig. 2a). Each peak was further identified by mass spectrometry. Figure 3 shows bar graphs of mass spectra of TMS-derivatives of α'-mycolic acid methyl esters from one of the strains of *M. smegmatis* (Takeo). As shown in Scheme 1, the general fragmentation of TMS-methyl mycolates on electron impact MS yielded characteristic ions useful for identification. The abundance of the $[M-15]^+$ ion (loss of methyl) and the $[M-90]^+$ ion (loss of trimethylsilanol) rather than molecular ion $[M]^+$, aided in determining the carbon and double bond number of whole molecules. Furthermore, the fragment ion $[A]^+$ (cleavage between the C_2–C_3 bond) and the ion $[A-90]^+$ (loss of trimethylsilanol) was prominent and

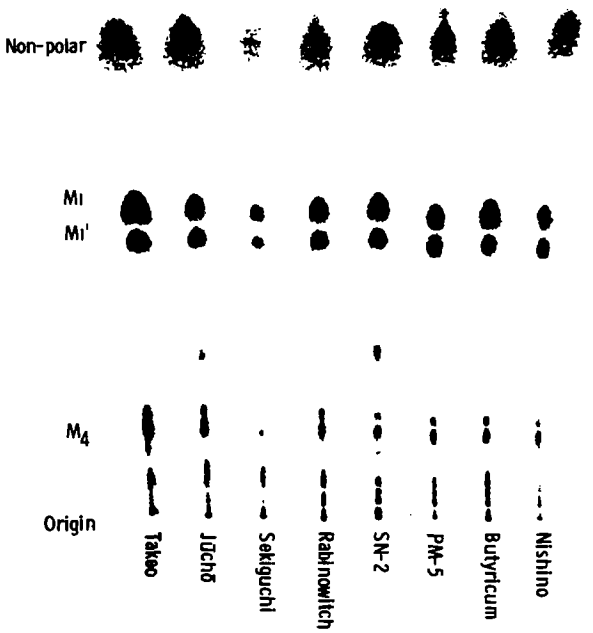


FIG. 1. Thin layer chromatograms of fatty acid methyl esters from eight strains of *M. smegmatis*, developed with n-hexane/diethyl ether (4:1, v/v).

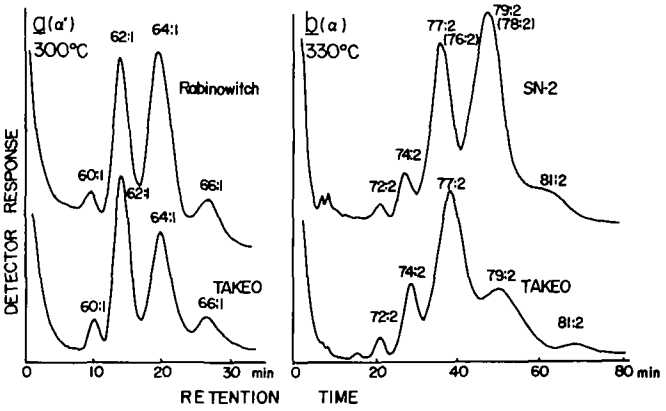


FIG. 2. Representative gas chromatograms of trimethylsilyl ether derivatives of methyl α' (a) and α (b)-mycolates from *M. smegmatis*. Numbers on each peak represent the carbon and double bond numbers as determined by GC–MS. Gas chromatography conditions are described in the text.

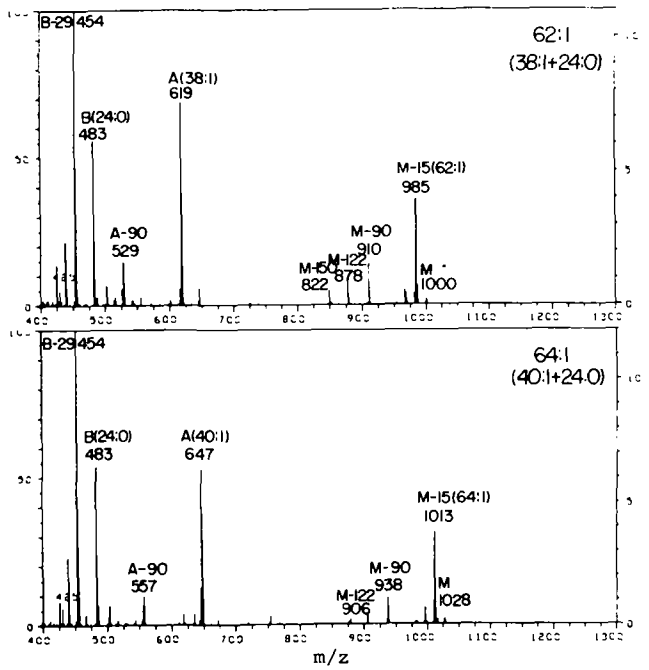
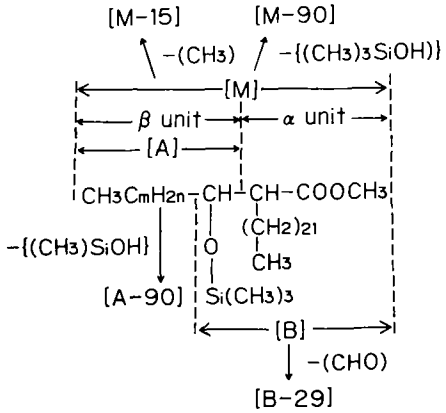


FIG. 3. Mass spectra of trimethylsilyl ether derivatives of methyl α'-mycolates of *M. smegmatis* (Takeo): $C_{62:1}$ (upper) and $C_{64:1}$ (lower), respectively.



SCHEME 1. General structure and mass fragmentation pattern of trimethylsilyl ether derivatives of methyl mycolates.

useful for the determination of the straight-chain alkyl moiety of the original mycolic acids. On the other hand, fragment ion $[B]^+$ (fission of C_3–C_4) and $[B\text{-}29]^+$ (probably loss of CHO) were very intense and useful for the identification of the α-branched-chain structure of mycolic acids. From the fragment ions $[M]^+$, $[M\text{-}15]^+$ and $[M\text{-}90]^+$, the original structure of α′-mycolates was determined as $C_{62:1}$ and $C_{64:1}$. Fragment ions $[A]^+$ and $[A\text{-}90]^+$ varied in the individual peaks of the gas chromatogram, which indicated that the straight-chain units varied from $C_{36:1}$ to $C_{42:1}$. On the other hand, the α-unit was $C_{24:0}$ among all molecular species of α′-mycolates because the fragment ions $[B]^+$ and $[B\text{-}29]^+$ were distinctively observed at *m/z* 483 and *m/z* 454, as shown in Table 1. After catalytic hydrogenation of methyl α′-mycolates with PtO_2, the sample was trimethylsilylated and analyzed again by GC–MS. The results are shown in the lower portion of Table 1. Each mass number of $[M\text{-}15]^+$, $[M\text{-}90]^+$ ions and the fragment ion $[A]^+$ of the straight-alkyl-chain (β) unit shifted up by two mass numbers. It was therefore confirmed that the α′-mycolic acids, up to C_{66}, from *M. smegmatis* had only one double bond in the straight-alkyl chain (β-unit). The fragment ions and straight-chain and α-alkyl-chain structures of individual molecular species of α′-mycolates are summarized in the upper part of Table 1. Table 2 shows the percentage composition of α′-mycolic acids from eight strains of *M. smegmatis* calculated from the peak areas in the gas chromatograms. Although there is minor quantitative variation in the percentage composition among the strains, the main components of α′-mycolates are $C_{62:1}$ and $C_{64:1}$ in all eight strains of *M. smegmatis*. The average carbon number of each strain was usually about 63, determined by the sum of the multiplication of carbon numbers by the percentages of the composition (carbon number × percentage).

The gas chromatographic pattern of the TMS-derivatives of methyl α-mycolates (M_1) from *M. smegmatis* is shown in Figure 2b, and similar patterns were found in seven strains. Typically, M_1 had two major peaks corresponding to C_{77} and C_{79} such as in the SN-2 strain. On the other hand, the Takeo strain had one large (C_{77}) and two small (C_{75} and C_{79}) peaks in the gas chromatogram. The mass spectra of individual TMS-methyl-mycolates on GC were recorded, and two are shown in Figure 4. As

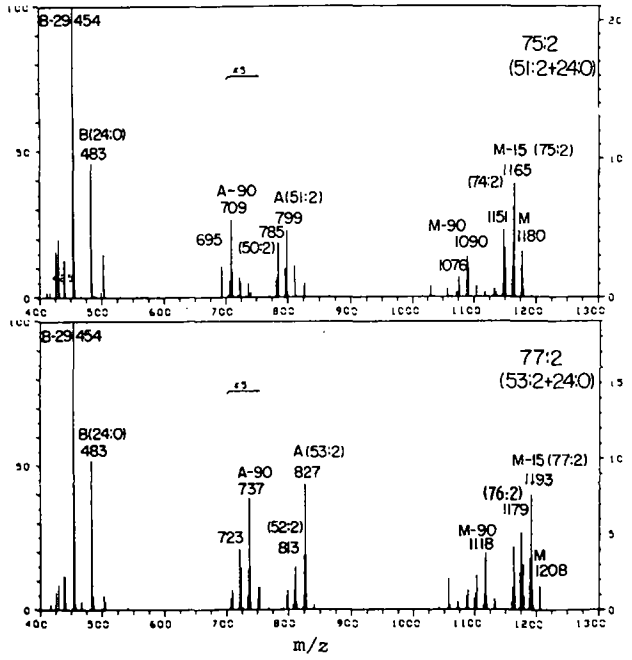


FIG. 4. Mass spectra of trimethylsilyl ether derivatives of methyl α-mycolates of the strain Takeo: $C_{75:2}$ (upper) and $C_{77:2}$ (lower), respectively.

TABLE 1

GC–MS Analysis of TMS-Methyl α′-Mycolate (M_1′) Before and After Hydrogenation (Takeo)

		$[M]^+$	$[M\text{-}15]^+$	$[M\text{-}90]^+$	$[A]^+$	$[A\text{-}90]^+$	β-unit	$[B]^+$	$[B\text{-}29]^+$	α-unit
Before	$C_{60:1}$	972	957	882	591	501	$C_{36:1}$	483	454	$C_{24:0}$
	$C_{62:1}$	1000	985	910	619	529	$C_{38:1}$	483	454	$C_{24:0}$
	$C_{64:1}$	1028	1013	938	647	557	$C_{40:1}$	483	454	$C_{24:0}$
	$C_{66:1}$	1056	1041	966	675	585	$C_{42:1}$	483	454	$C_{24:0}$
After	$C_{60:0}$	974	959	884	593	503	$C_{36:0}$	483	454	$C_{24:0}$
	$C_{62:0}$	1002	987	912	621	531	$C_{38:0}$	483	454	$C_{24:0}$
	$C_{64:0}$	1030	1015	940	649	559	$C_{40:0}$	483	454	$C_{24:0}$
	$C_{66:0}$	1058	1043	968	677	587	$C_{42:0}$	483	454	$C_{24:0}$

TABLE 2

α′-Mycolic Acid (M_1′) Composition of Various Strains of *M. smegmatis*

Carbon no.	PM-5	Rabino.	Takeo	Sekigu.	Jucho	SN-2	Nishino	Butyr.
$C_{60:1}$	0.6	1.2	5.5	2.1	1.5	3.2	0.6	1.6
$C_{62:1}$	41.2	43.6	42.7	47.0	45.8	54.9	57.7	59.9
$C_{64:1}$	47.6	50.6	48.4	45.8	48.7	40.5	39.8	37.8
$C_{66:1}$	10.6	4.7	3.5	5.2	3.9	1.4	1.6	0.6
Average carbon no.	63.4	63.2	63.1	63.1	63.0	62.8	62.7	62.7

previously described, the carbon and double bond number of the whole molecules, the straight-alkyl-chain and α-branched-chain unit were determined. In the α-mycolic acids of *M. smegmatis*, the total carbon number varied from C_{74} to C_{81} and two double bonds were located in the straight-alkyl chain (Table 3). We also confirmed the existence of two double bonds on the straight-alkyl-chain unit (β-unit) by mass spectrometric analysis before and after hydrogenation, as in the case of the α′-mycolates. When α-mycolates were analyzed after hydrogenation in neutral solvent, the mass spectrum showed four mass number increases in $[M-15]^+$ and $[A]^+$ ions (Table 3), indicating the presence of two double bonds in the straight-alkyl-chain unit. Moreover, mass chromatography of TMS methyl α-mycolates revealed that the odd-carbon-numbered mycolic acids overlapped with the one-less-(even-) carbon-numbered species in a single peak on the gas chromatogram. Judging from the relationship between log retention times and carbon numbers, the odd-carbon-numbered α-mycolates seemed to have one methyl branch in the straight-alkyl-chain unit. The odd-carbon-numbered acids were relatively higher than those of the even ones, as shown in Figure 5. These GC-MS results are summarized in Table 3. The molecular species composition of α-mycolates was calculated from the area percentages on the gas chromatograms (Table 4). Most strains had mainly odd-carbon-numbered mycolic acids, especially with C_{77} and C_{79}. On the other hand, only the Takeo strain possessed mainly a C_{77}-mycolic acid. The average carbon number of the α-mycolic acid of the Takeo strain was 76.3, differed from other strains that were close to 78.

The gas chromatographic profiles of TMS-derivatives of the polar mycolic acid TLC subclass (M_4) from two strains of *M. smegmatis* are shown in Figure 6. This sublcass of mycolates (M_4) occupies 2 to 5% of the total

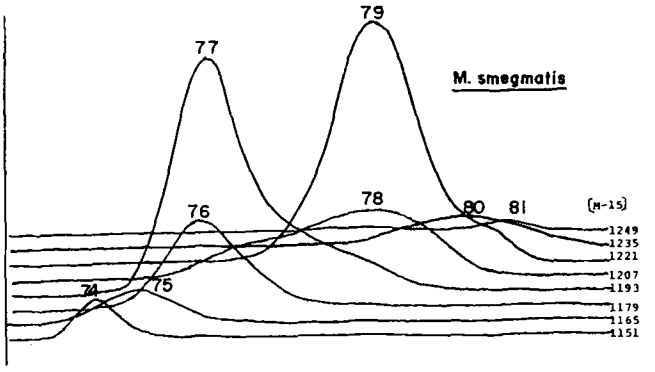


FIG. 5. Mass chromatograms of trimethylsilyl ether derivatives of methyl α-mycolates of *M. smegmatis*. $[M-15]^+$ ions corresponding to each carbon-numbered trimethylsilyl ether derivatives of dienoic methyl α-mycolates were monitored.

TABLE 3

GC-MS Analysis of TMS-Methyl α-Mycolate (M_1) Before and After Hydrogenation (Takeo)

		$[M]^+$	$[M-15]^+$	$[A]^+$	$[A-90]^+$	$[B]^+$	$[B-29]^+$	β-unit	α-unit
Before	$C_{74:2}$	1166	1151	785	695	483	454	$C_{50:2}$	$C_{24:0}$
	$C_{75:2}$	1180	1165	799	709	483	454	$C_{51:2}$	$C_{24:0}$
	$C_{76:2}$	1194	1179	813	723	483	454	$C_{52:2}$	$C_{24:0}$
	$C_{77:2}$	1208	1193	827	737	483	454	$C_{53:2}$	$C_{24:0}$
	$C_{78:2}$	1222	1207	841	751	483	454	$C_{54:2}$	$C_{24:0}$
	$C_{79:2}$	1236	1221	855	765	483	454	$C_{55:2}$	$C_{24:0}$
	$C_{80:2}$	1250	1235	869	779	483	454	$C_{56:2}$	$C_{24:0}$
	$C_{81:2}$	1264	1249	883	793	483	454	$C_{57:2}$	$C_{24:0}$
After	$C_{74:0}$	1170	1155	789	699	483	454	$C_{50:0}$	$C_{24:0}$
	$C_{75:0}$	1184	1169	803	713	483	454	$C_{51:0}$	$C_{24:0}$
	$C_{76:0}$	1198	1183	817	727	483	454	$C_{52:0}$	$C_{24:0}$
	$C_{77:0}$	1212	1197	831	741	483	454	$C_{53:0}$	$C_{24:0}$
	$C_{78:0}$	1226	1211	845	755	483	454	$C_{54:0}$	$C_{24:0}$
	$C_{79:0}$	1240	1225	859	769	483	454	$C_{55:0}$	$C_{24:0}$
	$C_{80:0}$	1254	1239	873	783	483	454	$C_{56:0}$	$C_{24:0}$
	$C_{81:0}$	1268	1253	887	797	483	454	$C_{57:0}$	$C_{24:0}$

TABLE 4

α-Mycolic Acid (M_1) Composition of Various Strains of *M. smegmatis*

Carbon no.	Nishino	SN-2	Jucho	Rabino.	PM-5	Sekigu.	Butyr.	Takeo
$C_{72:2,73:2}$	1.0	0.6	0.2	0.4	0.1	0.3	0.5	2.4
$C_{74:2,75:2}$	4.9	4.1	3.0	3.6	2.1	4.2	4.6	20.4
$C_{76:2,77:2}$	41.5	43.9	44.2	42.6	45.0	49.4	55.0	61.2
$C_{78:2,79:2}$	52.3	50.9	51.8	53.1	52.8	41.5	40.0	15.4
$C_{80:2,81:2}$	trace	0.5	0.8	0.4	trace	4.6	trace	trace
Average carbon no.	77.7	77.9	78.0	78.0	78.0	77.9	77.8	76.3

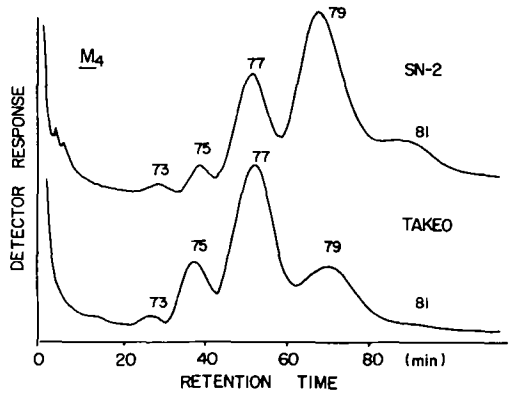


FIG. 6. Gas chromatograms of trimethylsilyl ether derivatives of methyl hydroxy mycolates (M_4) of two strains (SN-2 and Takeo). Numbers on each peak represent the carbon number as determined by GC-MS.

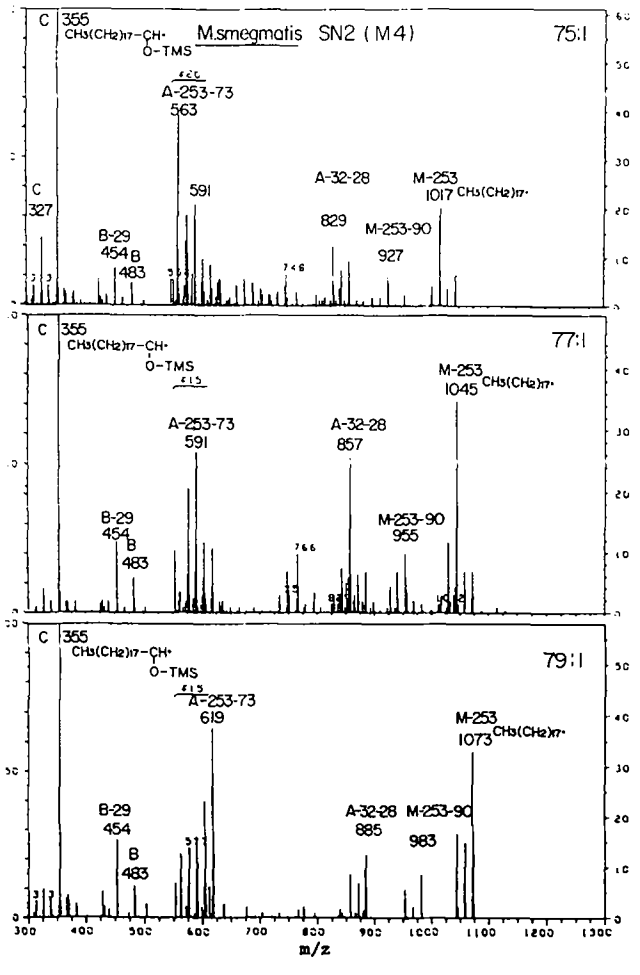


FIG. 7. Mass spectra of trimethylsilyl ether derivatives of methyl hydroxy mycolates (M_4) of the strain SN-2: from top to bottom, $C_{75:1}$, $C_{77:1}$, and $C_{79:1}$.

and is formed by acidic methylation after alkaline hydrolysis of the cellular lipids. Each GC profile of M_4 was similar to that of α-mycolates (Fig. 2b) in which only the Takeo strain had one major peak (C_{77}) and other strains had two major peaks (C_{77} and C_{79}). Mass spectra of three M_4-molecular species from the SN-2 strain are shown in Figure 7. Although the molecular ions cannot be detected, a very intense and characteristic mass ion was observed at *m/z* 355, due to the cleavage at the carbon atom, including O-TMS group, as shown in Figure 7. The other mass fragmentation patterns of polar mycolic acids were basically the same as those of α- or α'-mycolates. Therefore, these species were proposed as hydroxy mycolic acids with one double bond in the straight chain, ranging from C_{73} to C_{81}. The α-unit of M_4 was $C_{24:0}$, which was consistent again with those of the α- and α'-mycolates.

DISCUSSION

M. smegmatis is one of the representative species belonging to the rapidly growing, nonpathogenic and nonchromogenic group (31,38,39). *M. fortuitum* and *M. chelonae* also belong to the same group, but they are human pathogenic (40). Therefore, it is important to distinguish *M. smegmatis* from *M. fortuitum* and *M. chelonae*.

First, we attempted to identify the mycolic acids from eight strains of *M. smegmatis* by our TLC and GC-MS methods. The mycolic acids from all eight strains of *M. smegmatis* were separated by TLC into three subclasses, diunsaturated α-mycolates (M_1), lower molecular-weight monounsaturated α'-mycolates (M_1') and monounsaturated hydroxy mycolates (M_4) derived from epoxy mycolates. A similar pattern was reported by Minnikin et al. (35,36), who used repeated two-dimensional TLC for the analysis. Moreover, we examined the detailed structure and the composition of individual molecular species of mycolic acids by GC-MS analysis of TMS derivatives of mycolic acid methyl esters.

α'-Mycolates from *M. smegmatis* have 60, 62, 64 and 66 carbon atoms with one double bond, predominantly C_{62} and C_{64}. This result is consistent with the report of Wong and Gray (33). α-Mycolic acids commonly exist in all mycobacterial species, but α'-mycolates are the specific component in rapidly growing mycobacteria, such as *M. parafortuitum* (22), *M. chelonae* (41), *M. vaccae* (22) and *M. fortuitum* (22,36), but are absent in slow growing mycobacteria. α'-Mycolates from *M. vaccae, M. parafortuitum* and *M. chelonae* (41) were centered at $C_{58:1}$ and $C_{60:1}$, respectively. On the other hand, those from *M. fortuitum* were diunsaturated. The composition of α'-mycolates was essentially consistent among eight strains of *M. smegmatis*, but not among other species.

α-Mycolates from *M. smegmatis* were in common with other species of rapidly growing nonchromogenic mycobacteria, with respect to the general formulas $C_nH_{2n-4}O_3$ (n = 68-86) containing two double bonds in the straight-alkyl chain, and an α-unit of $C_{24:0}$ (22). It is characteristic in α-mycolates from *M. smegmatis* that the major carbon number is odd-numbered centering at C_{77} or C_{79}, which is due to the existence of one methyl branch in the even-carbon-numbered straight-alkyl chain. Wong and Gray (33) and Danielson and Gray (34) reported the presence of methyl-branched-chain α-mycolates in *M. smegmatis*, but they did not examine the relative quantity of odd- and even-carbon-numbered mycolates. In our previous paper (22), we found through mass chromatography that two types of odd-carbon-numbered-mycolic acids existed

TABLE 5

Properties and Mycolic Acid Subclass of Rapidly Growing *Mycobacteria*

		M. smegmatis	*M. chelonei*	*M. fortuitum*	*M. chitae*	*M. parafortuitum*	*M. vaccae*	*M. phlei*
Pigment production		−	−	−	−	+	+	+
Growth in <5 days		+	+	+	+	+	+	+
Mycolic acid subclass								
M_1 (α-)	CN	77,79	74,75	76∼80	76,77	74,76	74,76	74,76
	DB	(2)	(2)	(2)	(2)	(2)	(2)	(2)
M_1 (α'-)	CN	62,64	64	68	+	58,60	58,60	−
	DB	(1)	(1)	(2)	?	(1)	(1)	
M_2 (keto-)	CN	−	−	−	−	+	+	+
	DB							
M_2' (epoxy-)	CN	77,79	−	76∼79	77,79	−	−	−
	DB	(1)		(1)	(1)			
M_3 (dicarboxy-)	CN	−	−	−	−	56,58	56,58	60,62
	DB					(1)	(1)	(1)

CN, Major carbon number; DB, double bond; M_2', including M_4; +, positive or productive; −, negative or not productive.

in mycobacterial species. The first type appeared just between the even-carbon-numbered straight-alkyl-chain mycolates on gas chromatograms which was due to the presence of odd-carbon-numbered straight-alkyl chain or one or two cyclopropane rings. The other had almost the same retention time as that of the one-less-even-carbon-numbered molecules, as seen in *M. smegmatis, M. fortuitum, M. chelonae* and *M. chitae.* This would be due to the existence of one methyl-branch in the even-carbon-numbered straight-alkyl chain. We confirmed by mass spectrometry before and after hydrogenation, under very mild conditions with neutral solvents, that the main alkyl chain of α-mycolates from *M. smegmatis* had two double bonds and no cyclopropane ring because there was an increase of four mass numbers in the $[M\text{-}15]^+$ and $[A]^+$ ions. In contrast, when the α-mycolates of *M. vaccae,* which had two cyclopropane rings in the main alkyl chain, were hydrogenated in the same neutral solvent, no change was observed in the mass spectrum and the shift of mass ions and retention times was observed after hydrogenation in acetic acid (42). Because *M. fortuitum, M. chelonae* and *M. chitae* also have no cyclopropane rings in the skeleton structure of α-mycolates (42), one of the characteristics of nonchromogenic and rapidly growing mycobacteria would be likely to have one methyl branch and two double bonds without cyclopropane rings in α-mycolates. The average carbon number of α-mycolates from seven strains of *M. smegmatis* examined was about 78, but that of the Takeo strain was 76.3. Although the Takeo strain is close to *M. chitae* rather than *M. smegmatis* in molecular species of α-mycolates, we believe that the Takeo strain belongs to *M. smegmatis* and not to *M. chitae* because the composition of M_1' is essentially different than that of *M. chitae* (42).

The occurrence of epoxy mycolates in *M. fortuitum* was first reported by Daffe et al. (43). Successively, their characterization and distribution has been reported by Minnikin et al. (36,37) and Lévy-Frébault et al. (44), mainly using spectroscopic analyses, chemical transformations and degradations. However, epoxy mycolates are unstable under acidic conditions and it was shown (37) that epoxy mycolates are converted to dihydroxy or hydroxymethoxy mycolates through diol-type intermediates by acid methanolysis. We speculate that the oxygenated mycolates (M_4) in *M. smegmatis* are hydroxy mycolates produced from epoxy mycolates by the acidic methylation. The compositional profile of hydroxy mycolic acids (M_4) from eight strains of *M. smegmatis* is very similar to that of α-mycolic acids (M_1) from each strain. Although M_4 is a minor component in the mycolic acid subclass in some strains of *M. smegmatis,* it may be an important clue to clarify the biosynthesis or metabolism of this type of mycolate.

Table 5 summarizes the biological properties and mycolic acid subclasses of rapidly growing, nonchromogenic mycobacteria. *M. smegmatis* is closely related to *M. fortuitum, M. chelonae* and *M. chitae* in its physiological characteristics, and the profiles of chemical structures (subclass composition) of mycolic acids are shared. However, there is no species that has essentially the same profile of mycolic acid molecular species or subclass composition as *M. smegmatis.*

Using our TLC and GC–MS method for the analysis of mycolic acids, we can clearly distinguish *M. smegmatis* from *M. fortuitum, M. chelonae* and *M. chitae.*

REFERENCES

1. Anderson, R.J. (1929) *J. Biol. Chem. 85,* 351–354.
2. Stodola, F.H., Lesuk, A., and Anderson, R.J. (1938) *J. Biol. Chem. 126,* 505–513.
3. Asselineau, J., and Lederer, E. (1950) *Nature London 166,* 782–783.
4. Barbier, M., and Lederer, E. (1954) *Biochim. Biophys. Acta 14,* 246–258.
5. Lederer, E. (1967) *Chem. Phys. Lipids 1,* 294–315.
6. Etémadi, A.H., Okuda, R., and Lederer, E. (1964) *Bull. Soc. Chim. Fr. 4,* 868–870.
7. Minnikin, D.E., Alshamaony, L., and Goodfellow, M. (1975) *J. Gen. Microbiol. 88,* 200–204.
8. Bordet, C., and Michel, G. (1969) *Bull. Soc. Chim. Biol. 51,* 527–547.
9. Asselineau, J. (1961) *Biochim. Biophys. Acta 54,* 359–361.
10. Alshamaony, L., Goodfellow, M., and Minnikin, D.E. (1976) *J. Gen. Microbiol. 92,* 188–199.
11. Goodfellow, M., and Anderson, G. (1977) *J. Gen. Microbiol. 100,* 99–122.
12. Tomiyasu, I., and Yano, I. (1984) *Eur. J. Biochem. 139,* 173–180.

13. Alshamaony, L., Goodfellow, M., Minnikin, D.E., Bowden, G.H., and Hardie, J.M. (1977) *J. Gen. Microbiol. 98,* 205-213.
14. Collins, M.D., Goodfellow, M., and Minnikin, D.E. (1982) *J. Gen. Microbiol. 128,* 129-149.
15. Athalye, M., Noble, W.C., Mallet, A.I., and Minnikin, D.E. (1984) *J. Gen. Microbiol. 130,* 513-519.
16. Lederer, E., Adam, A., Ciorbaru, R., Petit, J.F., and Wietzerbin, J. (1975) *Mol. Cell Biochem. 7,* 87-104.
17. Durand, E., Welby, M., Lanéelle, G., and Tocanne, J.F. (1979) *Eur. J. Biochem. 93,* 103-112.
18. Retzinger, G.S., Meredith, S.C., Takayama, K., Hunter, R.L., and Kézdy, F.J. (1981) *J. Biol. Chem. 256,* 8208-8216.
19. Retzinger, G.S., Meredith, S.C., Hunter, R.L., Takayama, K., and Kézdy, F.J. (1982) *J. Immunol. 129,* 735-744.
20. Toriyama, S., Yano, I., Masui, M., Kusunose, M., and Kusunose, E. (1978) *FEBS Lett. 95,* 111-115.
21. Toriyama, S., Yano, I., Masui, M., Kusunose, E., Kusunose, M., and Akimori, N. (1980) *J. Biochem. 88,* 211-221.
22. Kaneda, K., Naito, S., Imaizumi, S., Yano, I., Mizuno, S., Tomiyasu, I., Baba, T., Kusunose, E., and Kusunose, M. (1986) *J. Clin. Microbiol. 24,* 1060-1070.
23. Yano, I., Kageyama, K., Ohno, Y., Masui, M., Kusunose, E., Kusunose, M., and Akimori, N. (1978) *Biomed. Mass Spectrom. 5,* 14-24.
24. Tomiyasu, I. (1982) *J. Bacteriol. 151,* 828-837.
25. Tomiyasu, I., Toriyama, S., Yano, I., and Masui, M. (1981) *Chem. Phys. Lipids 28,* 41-54.
26. Wada, H., Okada, H., Suginaka, H., Tomiyasu, I., and Yano, I. (1981) *FEMS Microbiol. Lett. 11,* 187-192.
27. David, H.L. (1977) *J. Gen. Microbiol. 101,* 99-102.
28. Matula, M., Mitchell, M., and Elbein, A.D. (1971) *J. Bacteriol. 107,* 217-222.
29. Winder, F.G., and Denneny, J.M. (1959) *Nature London 184,* 742.
30. Izumori, K., Rees, A.W., and Elbein, A.D. (1975) *J. Biol. Chem. 250,* 8085-8087.
31. Tsukamura, M., Mizuno, S., and Tsukamura, S. (1968) *Jpn. J. Microbiol. 12,* 151-166.
32. Wong, M.Y.H., Steck, P.A., and Gray, G.R. (1979) *J. Biol. Chem. 254,* 5734-5740.
33. Wong, M.Y.H., and Gray, G.R. (1979) *J. Biol. Chem. 254,* 5741-5744.
34. Dannielson, S.T., and Gray, G.R. (1982) *J. Biol. Chem. 257,* 12196-12203.
35. Minnikin, D.E., Hutchinson, I.G., and Calidicott, A.B. (1980) *J. Chromatogr. 188,* 221-223.
36. Minnikin, D.E., Minnikin, S.M., Hutchinson, I.G., Goodfellow, M., and Grange, J.M. (1984) *J. Gen. Microbiol. 130,* 363-367.
37. Minnikin, D.E., Minnikin, S.M., and Goodfellow, M. (1982) *Biochim. Biophys. Acta 712,* 616-620.
38. Runyon, E.H., Wayne, L.G., and Kubica, G.P. (1974) in *Bergey's Manual of Determinative Bacteriology* (Buchanan, R.E., and Gibbons, N.E., eds.) 8th edn., pp. 681-701, The Williams & Wilkins, Baltimore, MO.
39. Gordon, R.E., and Smith, M. (1953) *J. Bacteriol. 66,* 41-48.
40. Dresin, R.B., Scoggin, C., and Davidson, P.T. (1976) *Tubercle 57,* 49-57.
41. Minnikin, D.E., Minnikin, S.M., Goodfellow, M., and Stanford, J.L. (1982) *J. Gen. Microbiol. 128,* 817-822.
42. Kaneda, K., Imaizumi, S., Mizuno, S., Baba, T., Tsukamura, M., and Yano, I. (1988) *J. Gen. Microbiol. 134,* 2213-2229.
43. Daffé, M., Lanéelle, M.A., Puzo, G., and Asselineau, C. (1981) *Tetrahedron Lett. 22,* 4515-4516.
44. Lévy-Frébault, V., Daffé, M., Goh, K.S., Lanéelle, M.A., Asselineau, C., and David, H.L. (1983) *J. Clin. Microbiol. 17,* 744-752.

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Fatty Acids as Biological Markers for Bacterial Symbionts in Sponges[1]

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Analyses of fatty acids with carbon numbers between C_{12} and C_{22} are reported for five Great Barrier Reef sponges. These analyses indicate that phototrophic cyanobacterial symbionts (blue-green algae) present in three of the sponges are chemically distinct, whereas the other two sponges do not contain cyanobacterial symbionts. All the sponges contain other, nonphototrophic bacteria. The fatty acid analyses indicate that the nonphototrophic bacterial populations present in the different sponges are distinct in both their chemical compositions and their abundances. Nonphototrophic bacteria are estimated to account for between 60 and 350 μg/g (extractable fatty acids:tissue wet weight), whereas cyanobacteria account for between 10 and 910 μg/g. One sponge (*Pseudaxinyssa* sp.) contains a relatively large amount of the isoprenoid acid, 4,8,12-trimethyltridecanoic acid; this acid is presumed to be derived from phytol, a degradation product of chlorophyll. This sponge also contains relatively large amounts of the nonmethylene interrupted fatty acid, octadeca-5,9-dienoic acid. Analyses of interior and cyanobacteria-rich surface tissues of this sponge indicate that these two acids are probably not associated with the symbiotic cyanobacteria.

***Lipids 23,* 1139–1145 (1988).**

Marine sponges are rich sources of C_{24}-C_{30} fatty acids (1–3), in addition to the shorter C_{14}-C_{22} fatty acids typically found in other organisms. Marine sponge phospholipids also include numerous branched fatty acids, where extra methyl groups can appear either at a middle or terminal position (4,5) (Fig. 1). Litchfield and coworkers have demonstrated that sponges synthesize these long fatty acids primarily by the extension of the short fatty acids, based on their incorporation of labeled acetate into such acids (1), in the manner in which we have shown branched short chain acids serve as primers for long-chain branched acids in the sponge *Jaspis stellifera* (6). Bacteria are the only organisms commonly reported to produce large amounts of iso-, anteiso-, cyclopropyl and monomethyl branched fatty acids (7,8), although some bacteria biosynthesize only straight-chain fatty acids. These latter bacteria have been divided into four chemical groups characterized solely by the presence of the acids 16:1(9), 18:1(11), 16:1(9t) and 18:1(11t), respectively (9).

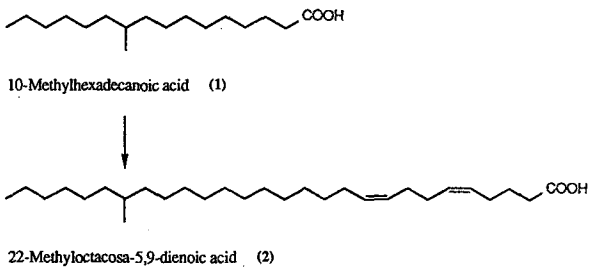

FIG. 1. A short-chain, branched fatty acid 1 assumed to be the precursor of the long-chain, $\Delta^{5,9}$-branched acid 2 in the marine sponge *Aplysina fistularis* (4).

The origin of such bacterial-type fatty acids within sponge tissue is presumed to be of microbial origin, and can be either dietary, from their diet of planktonic microorganisms (<50 μm), or acquired from the large populations of microorganism symbionts found within the sponge body. The intercellular matrix of most terrestrial animals is sterile; in contrast, many marine organisms such as sponges and ascidians often have cyanobacteria (blue-green algae), nonphototrophic bacteria, prochlorophytes and algae associated with this matrix (10–14). Some sponges have been observed to contain very few nonphototropic bacterial symbionts (15–17), whereas in others such bacteria may occupy 40% or more of the whole sponge volume, including as many as seven morphologically different bacterial species (16,18). Similarly, cyanobacterial symbionts can be absent, present or very abundant in sponges (13,14). The presence of cyanobacteria within a particular sponge can readily be inferred by the detection of undegraded chlorophylls in a lipid extract of the sponge tissue where algal epibionts are not present; no such simple procedure is available for the detection and estimation of nonphototrophic bacterial symbionts.

Geochemical studies by Johns and coworkers have demonstrated the usefulness of fatty acids as biological markers for algae and bacteria in marine sediments (7,19,20) and for prochlorophyte symbionts in an ascidian (21). Extensive studies of the fatty acids present in sponges have been reported by Bergquist and coworkers (2,3); however, these reports were not sufficiently quantitative to be useful for biological marker identification. Early studies suggested that certain fatty acids present in sponges originate only from bacteria (4,22). Recent studies demonstrate that bacteria isolated either mechanically or by culture from several host sponges contain short-chain fatty acid precursors (C. Djerassi and coworkers, unpublished data) that can be efficiently utilized for the biosynthesis of long-chain fatty acids (6,23,24). In the present study, we extend the previous microorganism biological marker work (21) and adapt a recently developed bacterial biological marker technique based on fatty acid content (9) to the estimation of cyanobacterial and nonphototrophic bacterial abundance in sponges.

EXPERIMENTAL

Collection and extraction of sponge tissues. Small samples (0.5–2 g fresh weight) of five sponges were collected in the central portion of the Australian Great Barrier Reef

[1]This manuscript is Part 21 in the Stanford series "Phospholipid Studies of Marine Organisms." For Part 20, see reference 42.

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Abbreviations: TMTD, 4,8,12-trimethyltridecanoic acid; ECL, equivalent chain length; GC-MS, gas chromatography-mass spectrometry.

(Davies Reef), including three phototrophic, cyanobacteria-containing species (*Dysidea herbacea* [O. Dictyoceratida], *Phyllospongia papyracea* [O. Dictyoceratida] and *Pseudaxinyssa* sp. [O. Axinellida, Australian Museum specimen Z4988]) and two nonphototrophic species (*Phakellia aruensis* [O. Axinellida] and *Rhopaloeides odorabile* [O. Dictyoceratida, previously referred to as *Spongia* sp {25}]) (26,27).

Samples were immediately placed in solvent (chloroform/methanol, 1:1, v/v, plus 0.05% pyridine), with static lipid extraction (24 hr) proceeding in the dark at room temperature. Decantation was followed by reextraction of the sponge residue by grinding in two further changes of solvent. The combined extracts contained >95% of the lipid content of the sponges. An aliquot of this lipid extract was saponified and the resultant fatty acids were converted to methyl esters by standard procedures (28,29).

In the later examination of the distribution of fatty acids between interior and cyanobacteria-rich surface tissues, a sample of *Pseudaxinyssa* sp. was collected from nearby Rib Reef. This sample was dissected into two portions: surface tissues to a depth of 0.5 mm, including exopinacoderm and a distinct reddish-brown band of cyanobacteria, and interior tissues (cream colored). Each portion was lyophilized prior to extraction and the isolated phospholipids were converted to methyl esters, followed by subsequent gas chromatographic analysis. Electron microscopy was used to confirm the large volume of cyanobacteria within the surface tissue fraction and the almost complete absence of cyanobacteria within the interior tissue fraction.

Fatty acid analyses. Fatty acid methyl esters were analyzed on both apolar (50 m × 0.2 mm, SP2100, Hewlett-Packard, Palo Alto, CA) and polar (50 m × 0.2 mm, Superox 0.1, S.G.E. Australia) capillary columns, and the raw data from electronic integration were interpreted using our previously reported computer programs (30). Structures were confirmed by chromatographic comparison with authentic standards where available, by comparison of equivalent chain lengths (ECL) for the methyl esters with those of secondary standards and by capillary gas chromatography–mass spectrometry (GC–MS) (HP 5970A ion selective detector coupled to an HP 5790B gas chromatograph). Long-chain ($\geq C_{24}$) demospongic acids, including previously identified nonmethylene interrupted di- and triunsaturated fatty acids (characteristic of certain sponges) (4,22,31–38) were also identified for these sponges, but are not considered in the present study.

Bacterial community structure analysis. The respective contributions of symbiotic bacteria were determined according to the methods of Johns et al. (21) and Gillan and Hogg (9), wherein the fatty acids extracted from sponge tissue are assumed to originate primarily from three sources—cell membranes of the sponge, nonphototrophic bacteria or cyanobacteria. The minimum abundance of any specific acid in a suite of sponge samples was used as an estimation of the sponge contribution to the abundance of that acid in the other sponges. This method should be especially accurate for estimation of cyanobacterial contributions for our suite of sponge samples as two of these species do not contain cyanobacteria. This method, however, requires that one assumption be made, namely that sponge membrane fatty acid compositions vary within a limited range. Work in our Stanford laboratory currently seeks to test this assumption via the laborious procedure of isolating pure sponge membranes in sufficient quantity for fatty acid analyses (39–42).

RESULTS AND DISCUSSION

Lipids extracts. Three of the five sponges yielded green extracts on treatment with chloroform/methanol. Visible spectroscopy and thin layer chromatography confirmed the presence of chlorophyll-a in these extracts. As expected, the neutral lipid fraction from saponification of the extracts from these three sponges (*D. herbacea, P. papyracea* and *Pseudaxinyssa* sp.) contained appreciable amounts of phytol (3, Fig. 2), indicating the presence of chlorophyll, presumably largely of cyanobacterial origin. In contrast, lipid extracts from the other two sponges (*P. aruensis* and *R. odorabile*) did not exhibit a visible absorption maximum in the 600–700 nm region of their spectra and did not yield phytol (3) on saponification, indicating the absence of cyanobacteria.

In *Pseudaxinyssa* sp., the chlorophyll was almost exclusively associated with the surface tissues of the sponge, as contrasted to the interior tissues of the sponge, in accordance with our microscopic observations. Electron microscopy indicated that cyanobacteria were present in appreciable amounts only in the surface tissues of the sponge.

Fatty acid compositions. The fatty acid compositions of the five sponges are reported in Table 1 where double bonds for diunsaturated and polyunsaturated fatty acids are methylene-interrupted unless specifically stated otherwise. In general, only the position of the carboxyl terminal double bond is stated in these cases. None of the samples examined were markedly similar to any of the others. The three cyanobacteria-containing sponges (*D. herbacea, P. papyracea* and *Pseudaxinyssa* sp.) yielded relatively much

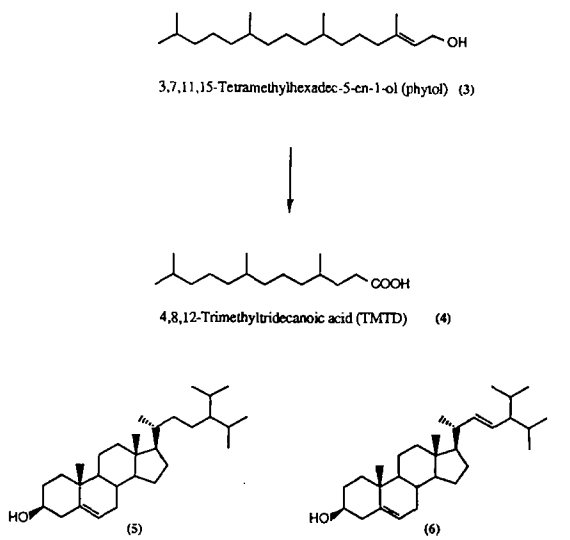

FIG. 2. Unconventional, highly branched sterols 5 and 6 from *Pseudaxinyssa* sp. and 4,8,12-trimethyltridecanoic acid (TMTD) (4), a highly branched fatty acid derived from phytol and isolated as the major fatty acid from this sponge.

TABLE 1

The Abundances of Monocarboxylic Acids in Five Great Barrier Reef Sponges

Fatty acid[a] (μg/g wet wt)	Sponge species				
	With cyanobacteria			Without cyanobacteria	
	D. herbacea	*P. papyracea*	*Pseudaxinyssa* sp.	*P. aruensis*	*R. odorabile*
12:0	1	2	1	—	—
13:0	3	2	6	—	—
i14:0	—	—	—	—	2
14:1(7)	1	—	1	—	—
14:1(9)	5	1	5	—	—
14:0	17	75	20	11	11
TMTD	—	—	54	—	—
i15:0	2	13	6	1	71
a15:0	1	16	6	—	22
15:0	5	7	5	3	7
i16:0	3	7	2	5	15
16:1(7)	9	3	4	2	2
16:1(9)	16	232	75	13	5
16:0	352	485	173	60	49
Me16:0	—	6	11	17	34
i17:1(9)	—	—	5	—	18
i17:0	3	19	3	2	12
a17:0	3	27	5	—	14
17:1(9)	—	2	—	3	—
cy17:0(9)	—	12	—	3	2
17:0	12	28	6	3	4
18:2(5,9)	4	13	78	11	1
18:3(6)	114	—	—	1	2
18:4(6)	3	1	3	4	—
18:2(9)	50	3	3	7	—
18:3(9)	—	—	2	2	—
18:1(9)	153	22	122	12	3
18:1(11)	37	149	28	8	15
18:0	271	72	28	21	29
Me18:0	—	—	7	10	54
i19:0	—	—	—	1	2
a19:0	—	7	3	—	1
cy19:0(11)	—	7	5	1	8
19:0	2	7	9	1	2
20:4(5)	—	—	4	37	2
20:5(5)	2	2	2	7	—
20:1(11)	3	5	60	3	1
20:1(13)	4	8	17	2	1
20:0	14	17	14	3	3
21:0	2	5	2	—	3
22:5(4)	—	2	1	8	—
22:6(4)	1	16	2	16	1
22:1(13)	5	9	10	9	3
22:1(15)	4	91	4	7	1
22:0	4	14	2	2	2

[a]For diunsaturated and polyunsaturated fatty acids, all double bonds are methylene interrupted unless specifically stated otherwise. In general, only the position of the carboxyl terminal double bond is stated in these cases. TMTD = 4,8,12-trimethyltridecanoic acid; Me16:0 and Me18:0 refer to mixtures of monomethyl-branched C_{17} and C_{19} acids, respectively; abbreviations for iso, anteiso and cyclopropyl acids are i, a and cy, respectively.

larger amounts of fatty acids on extraction, particularly straight-chain saturated and monounsaturated fatty acids. Indeed, these three sponges contained, on average, ca. 900 μg/g (wet wt) of these acids compared to only 150 μg/g in the two nonphototrophic sponges (*P. aruensis* and *R. odorabile*). In contrast, no such clear distinction in branched-chain fatty acid distribution was observed for these sponges according to cyanobacterial content. The cyanobacteria-free sample of *R. odorabile* yielded the greatest amount of branched-chain fatty acids, whereas the sample of cyanobacteria-rich *D. herbacea* yielded the least. Only one sponge, *Pseudaxinyssa* sp., contained an appreciable amount of the isoprenoid fatty acid, 4,8,12-trimethyltridecanoic acid (TMTD) (4).

The fatty acid compositions of the interior and cyanobacteria-rich surface portions of *Pseudaxinyssa* sp. are reported in Table 2. The surface tissues were enriched in the 16:1(9), 16:0, 18:3(6), 18:1(9) and 18:1(11) acids,

TABLE 2

Relative Abundances of Monocarboxylic Acids in Surface and Interior Tissues of *Pseudaxinyssa* sp.[a]

Fatty acid[b] (μg/g wet wt)	Interior tissues	Surface tissues	Ratio surface/interior
14:1(9)	10	9	0.9
14:0	12	10	0.8
TMTD	100	100	1.0
i15:0	4	3	0.8
a15:0	4	3	0.8
15:0	3	3	1.0
16:2(5,9)	6	10	1.7
16:1(9)	37	221	6.0
16:0	75	366	4.9
17:0	4	4	1.0
18:2(5,9)	67	149	2.2
18:3(6)	6	30	5.0
18:1(9)	55	284	5.2
18:1(11)	26	72	2.8
18:0	16	21	1.3
cy19:9(11)	7	4	0.6
19:0	10	12	1.2
20:2(5,9)	37	42	1.1
20:1(11)	65	73	1.1
20:1(13)	26	33	1.3
20:0	15	13	0.9
22:5(4)	13	10	0.8
22:6(4)	19	12	0.6

[a]Results are normalized to TMTD = 100. Interior tissues lack cyanobacteria, whereas surface tissues (<0.5 mm) contain large numbers of cyanobacteria.

[b]For format and abbreviations used, see footnote, Table 1.

suggesting that these acids are of cyanobacterial origin.

Cyanobacterial symbionts. Cyanobacteria have been reported to biosynthesize fatty acids in the range C_{14} to C_{18}, with varying degrees of unsaturation (up to tetraenoic acids) (43,44). In addition, it has been shown that the 18:1 isomer produced by cyanobacteria is 18:1(9) (45). Table 3 lists the residual abundances of potential cyanobacterial acids calculated by subtraction of the minimum observed abundance in any of the five specimens. The three species noted for the presence of cyanobacteria, as indicated by the presence of chlorophyll-a, yielded large residual amounts of these acids, whereas the two species without cyanobacterial symbionts yielded very low residual acid abundances. The 16:1(9) acid was found to be the major cyanobacterial acid present in these three cyanobacteria-rich sponges.

Studies employing electron microscopy reveal that *D. herbacea* contains a colonial (multicellular) cyanobacterium similar to *Phormidium spongeliae,* whereas *P. papyracea* and *Pseudaxinyssa* sp. yield a solitary (unicellular) cyanobacterium similar to *Aphanocapsa feldmanni* (10,46). The presence of linoleic (18:2(9)) and γ-linolenic acids (18:3(6)) in the residual acids of *D. herbacea,* but not the other sponges, is thus consistent with the earlier observation that colonial cyanobacteria usually produce polyenoic acids, whereas solitary species usually do not (8,43,44). This result also indicates that fatty acid analyses can potentially be used to screen sponge tissue samples for the presence of different cyanobacterial symbionts; for example, *P. papyracea* and *Pseudaxinyssa* sp. have different residual acid compositions likely indicating the presence of different cyanobacterial populations. The cyanobacteria from *P. papyracea* contain predominantly 16:0 (53%), 16:1(9) (24%), 14:0 (8%) and 18:0 (6%) fatty acids, but the cyanobacteria from *Pseudaxinyssa* sp. contain much more 18:1(9) (35% cf. 2% for *P. papyracea*) and relatively much less 14:0 (3%) and 18:0 (2%). This marked chemical difference cannot be explained solely by compositional variation within a cyanobacterial species; rather, different cyanobacterial species must be present.

The summed fatty acid contribution from cyanobacteria listed in Table 3 is a direct measure of the cyanobacterial biomass present in the sample of sponge tissue analysed. Indeed, because the abundance of fatty acids in cyanobacteria typically varies within the limited range of 3–5% (dry wt basis) (47) during one season, the fatty acid contribution can be used to calculate the biomass of cyanobacteria present. However, most studies on sponges report symbiont biomasses in terms of percentage tissue volume, because this is relatively easily

TABLE 3

Fatty Acids Attributable to Cyanobacteria in the Total Acids of Five Marine Sponges[a]

Fatty acid[a] (μg/g wet wt)	Host sponge				
	With cyanobacteria			Without cyanobacteria	
	D. herbacea	*P. papyracea*	*Pseudaxinyssa* sp.	*P. aruensis*	*R. odorabile*
14:0	6	64	9	—	—
16:0	303	436	124	11	—
16:1(9)	11	227	70	8	—
17:0	9	25	3	—	1
18:0	250	51	7	—	8
18:1(9)	150	19	119	9	—
18:2(9)	50	3	3	7	—
18:3(6)	114	—	—	1	2
Σ	913	825	335	35	11

[a]Cyanobacterial fatty acids are considered to be the residual abundance of acids calculated by subtraction of the minimum observed abundance of each acid in any of the five specimens. Only those acids with residual concentrations greater than 10 μg/g are listed.

estimated by electron microscopy. Although not advocating this practice, the data in Table 3 can be converted to this format with an assumption of the cyanobacterial water content; if the cyanobacterial water content is assumed to be 80%, then the symbiotic cyanobacteria volume contribution to the whole sponge volume can be calculated to vary from 3.4 to 9.1% (w/w) for each of the three cyanobacteria-containing sponges considered in this study.

Nonphototrophic bacterial symbionts. In an earlier study, Gillan and Hogg (9) proposed a method for nonphototrophic bacterial biomass estimation based on the abundances of characteristic bacterial acids. In this study, we cannot use the 16:1(9) abundance to estimate the biomass of the nonphototrophic bacterial group that produces this acid due to major production of this acid by cyanobacteria. In addition, we did not detect the *trans* acids, 16:1(9t) and 18:1(11t), in the sponge analyses. However, several isomers of both the C_{16} and C_{18} mid-chain, monomethyl-branched fatty acids (principally, 9- and 10-methyl 16:0, and 11- and 12-methyl 18:0) were present in most of the sponges. These latter acids have been reported in bacteria, especially sulfate-reducing bacteria (48). As in the case of cyanobacterial estimation, we have estimated the abundances of nonphototrophic bacterial acids due to in situ bacterial symbionts as the residual acid abundances (Table 4).

Two sponges, *P. papyracea* and *R. odorabile*, yielded much larger abundances of nonphototrophic bacterial acids than the other three sponges. However, the predominant bacteria in these two sponges are markedly different: *R. odorabile* contains mainly monomethyl-branched 16:0 and 18:0 acid-producing bacteria and iso/anteiso acid-producing bacteria, whereas *P. papyracea* contains largely bacteria with vaccenic acid (18:1[11]) (Table 4). *Pseudaxinyssa* sp. contained a mixture of these bacteria, but the total abundance was approximately one-fourth less, whereas *D. herbacea* and *P. aruensis* contained only traces of bacteria. The distribution of bacterial acids in *D. herbacea* was similar to that observed in *P. papyracea*.

Because branched acid-producing bacteria also produce small amounts of straight-chain acids, the summed abundances in Table 4 are an underestimate of the actual bacterial fatty acid contribution. A better estimate can be obtained by application of the equations published recently (9); these equations do not, however, account for methyl branched 16:0 and 18:0 producing bacteria. The estimated bacterial symbiont fatty acid contribution reported in Table 4 is based on the value derived by application of the equations plus twice the combined abundance of methyl branched 16:0 and 18:0 (as a first approximation it is assumed that methyl branched acids account for 50% of the total acids in these chemotypes). This calculated, nonphototrophic bacterial contribution varies between 50 μg/g wet wt and 350 μg/g wet wt for the tissue samples examined in this study. For comparison with electron microscopy studies, the approximate tissue volume contributions for bacteria in each of the five specimens can be calculated with the assumption that fatty acids represent 1% wet wt of the sponge (8). In this way, nonphototrophic bacteria represent ca. 1% (for cyanobacteria-containing species) or 3.5% (for cyanobacteria-free species) of the tissue volume of the host sponge, suggesting possible competition for tissue space between these two types of bacteria.

TABLE 4

Fatty Acids Attributable to Nonphototrophic Bacteria in the Total Fatty Acids of Five Marine Sponges[a]

Fatty acid[b] (μg/g wet wt)	Sponge species				
	D. herbacea	*P. papyracea*	*Pseudaxinyssa* sp.	*P. aruensis*	*R. odorabile*
i14:0	—	—	—	—	2
i15:0	1	12	5	—	70
a15:0	1	16	6	—	22
i16:0	1	5	—	3	13
Me16:0	—	6	11	17	34
i17:1(9)	—	—	5	—	18
i17:0	1	17	1	—	10
a17:0	3	27	5	—	14
cy17:0(9)	—	12	—	3	2
18:1(11)	29	141	20	—	7
Me18:0	—	—	7	10	54
i19:0	—	—	—	1	2
a19:0	—	7	3	—	1
cy19:0(11)	—	7	5	1	8
Σ	36	250	68	35	257
Estimated total bacterial fatty acid contributions:	56	347	100	64	343

[a]Bacterial residual acids are considered to be the residual abundance of acids calculated by subtraction of the minimum observed abundance of each acid in any of the five specimens. Estimated total bacterial contribution to acids is calculated according to methods described in the text.

[b]For format and abbreviations used, see footnote, Table 1.

TABLE 5

Calculated Tissue Volumes Contributed by Cyanobacteria and Nonphototrophic Bacteria for Five Great Barrier Reef Sponges

Sponge	Fatty acid contribution μg/g wet wt (% Tissue Volume)		
	Cyanobacteria	Other bacteria	Total
Dysidea herbacea	913 (9.1)	56 (0.6)	969 (9.7)
Phakellia aurensis	35 (0.0)	64 (0.6)	99 (0.6)
Phyllospongia papyracea	825 (8.3)	347 (3.5)	1172 (11.8)
Pseudaxinyssa sp.	335 (3.4)	100 (1.0)	435 (4.4)
Rhopaloeides odorabile	11 (0.0)	343 (3.4)	354 (3.4)

4,8,12-Trimethyltridecanoic acid. Pseudaxinyssa sp. was readily distinguishable from the other sponges due, among other factors, to the presence of a large abundance of the fatty acid 4,8,12-trimethyltridecanoic acid (TMTD) (4) (Fig. 2). This acid is an isoprenoid acid that is derived by degradation of phytol (3), which, in turn, is derived from chlorophyll. The high concentration of this acid in the interior, cyanobacteria-free tissues of this sponge (16.0%) vs that observed for surface tissues (6.7%) suggests that this phospholipid acid is one of the major sponge cell membrane components. In fact, recent work has established that TMTD (4) is present in the membranes of *Pseudaxinyssa* sp. (40). The TMTD in the sponge cells may be derived from degradation of the cyanobacterial chlorophyll phytol (Fig. 2), possibly with the assistance of other bacterial species, but a dietary origin cannot be excluded. It is unlikely that the sponge ingests live symbiotic cyanobacteria because sponges apparently have mechanisms to avoid ingestion of bacterial symbiont species (49).

This is the first case in which such a highly branched fatty acid appears to function within cell membranes, raising further questions about the structure and function of sponge membranes that are already noted for exceedingly long phospholipid fatty acids that are theoretically incompatible with normal membrane function (39,50). The presence of this highly branched acid as a major acid in the sponge cell membrane may be related to the extremely unusual sterol composition of *Pseudaxinyssa* sp. (41,42, 51-54), as suggested by Walkup et al. (4) for another sponge. Over 98% of the sterol mixture consists of the highly branched C_{30} sterols, 24-isopropyl cholesterol (5) and its 22-dehydro- analog (6) (Fig. 2). TMTD also occurs as a major fatty acid in the sterol esters within the interior portions of the sponge, whereas the cyanobacterial fatty acids predominate in the surface portion of the sponge. It should be noted, however, that the whole sponge *Spheciospongia vesparium* also contains large amounts of TMTD, but has a very simple sterol composition of mainly cholesterol and sitosterol (55).

Biosynthesis of fatty acids. The high amount of the 18:2($\Delta^{5,9}$) acid, a nonmethylene interrupted fatty acid unknown in bacteria, suggests that the $\Delta^{5,9}$-dehydrogenase enzyme of the sponge is not only specific for the long demospongic acids, but that the typical $\Delta^{5,9}$-unsaturation pattern (1) can be introduced into shorter fatty acid analogs as well. Thus, the 18:1(Δ^{9}) acid, contributed by symbiotic cyanobacteria, may be further desaturated to the 18:2($\Delta^{5,9}$) acid. This latter fatty acid has been isolated from at least two sponges (5,34); its lower homolog, 16:2($\Delta^{5,9}$), which has never before been reported for bacteria, was recently reported as a major constituent of the sponge *Chondrilla nucula* (56).

CONCLUSIONS

Fatty acid analyses have been shown to distinguish different symbionts present in marine sponges and to provide estimates of the bacterial symbiont abundances. According to fatty acid content, three of the sponges examined in this study were shown to contain cyanobacterial symbionts, whereas all the sponges contained nonphototrophic bacteria; Table 5 summarizes the estimated biomass data for each bacterial class in the five sponge species examined. Although this method sheds light on the contributions of fatty acids by bacterial symbionts in sponges, this method should be cautiously applied for estimation of fatty acid contributions by symbionts because a detailed analysis of the fatty acid composition of isolated (and possibly also cultured) bacterial symbionts and pure isolated sponge cell membranes should be used to unambiguously solve the problem. Some work along these lines has recently been reported (42).

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REFERENCES

1. Litchfield, C., Greenberg, A.J., Noto, G., and Morales, R.W. (1976) *Lipids 11*, 567-570.
2. Bergquist, P.R., Lawson, M.P., Lavis, A., and Cambie, R.C. (1984) *Biochem. Syst. Ecol. 12*, 63-84.
3. Lawson, M.P., Bergquist, P.R., and Cambie, R.C. (1984) *Biochem. Syst. Ecol. 12*, 375-393.
4. Walkup, R.D., Jamieson, G.C., Ratcliff, M.R., and Djerassi, C. (1981) *Lipids 16*, 631-646.
5. Dasgupta, A., Ayanoglu, E., and Djerassi, C. (1984) *Lipids 19*, 768-776.
6. Carballeira, N., Thompson, J.E., Ayanoglu, E., and Djerassi, C. (1986) *J. Org. Chem. 51*, 2751-2756.
7. Perry, G.J., Volkman, J.K., Johns, R.B., and Bavor, H.J. (1979) *Geochim. Cosmochim. Acta 43*, 1715-1725.

8. Gillan, F.T. (1981) Ph.D. Thesis, University of Melbourne, Melbourne, Australia.
9. Gillan, F.T., and Hogg, R.W. (1984) *J. Microbiol. Methods 2,* 275-293.
10. Sara, M. (1971) *Mar. Biol. 11,* 214-221.
11. Lewin, R.A., and Cheng, L. (1975) *Phycologia 14,* 149-152.
12. Newcomb, E.H., and Pugh, T.D. (1975) *Nature 253,* 533-534.
13. Vacelet, J. (1982) *Proc. 4th Int. Coral Reef Symp. 2,* 713-719.
14. Wilkinson, C.R. (1980) in *Endocytobiology: Endosymbiosis and Cell Biology* (Schwemmier, W., and Schenck, H.E.A., eds.), Vol. 1, pp. 553-563, Walter de Gruyter & Co., Berlin.
15. Vacelet, J., and Donadey, C. (1977) *J. Exp. Mar. Biol. Ecol. 30,* 301-314.
16. Wilkinson, C.R. (1978) *Mar. Biol. 49,* 169-176.
17. Mackie, G.O., and Singla, C.L. (1983) *Phil. Trans. Roy. Soc. Lond. B301,* 365-400.
18. Vacelet, J. (1975) *J. Microsc. Biol. Cell 23,* 271-288.
19. Volkman, J.K., and Johns, R.B. (1977) *Nature 267,* 693-694.
20. Volkman, J.K., Johns, R.B., Gillan, F.T., Perry, G.J., and Bavor, H.J., Jr. (1980) *Geochim. Cosmochim. Acta 44,* 1133-1143.
21. Johns, R.B., Nichols, P.D., Gillan, F.T., Perry, G.J., and Volkman, J.K. (1981) *Comp. Biochem. Physiol. 69B,* 843-849.
22. Ayanoglu, E., Walkup, R.D., Sica, D., and Djerassi, C. (1982) *Lipids 17,* 617-625.
23. Raederstorff, D., Shu, A.Y.L., Thompson, J.E., and Djerassi, C. (1987) *J. Org. Chem. 52,* 2337-2346.
24. Hahn, S., Stoilov, I.L., Ha, T.B., Raederstorff, D., Doss, G.A., Li, H.-T., and Djerassi, C. (1988) *J. Am. Chem. Soc.,* in press.
25. Thompson, J.E., Murphy, P.T., Bergquist, P.R., and Evans, E.A. (1987) *Biochem. Syst. Ecol. 15,* 595-606.
26. Wilkinson, C.R. (1983) *Science 219,* 410-412.
27. Wilkinson, C.R., and Trott, L.A. (1985) *Proc. 5th Intl. Coral Reef Congr. 5,* 125-130.
28. Johns, R.B., Nichols, P.D., and Perry, G.J. (1979) *Phytochemistry 18,* 799-802.
29. Johns, R.B., Nichols, P.D., and Perry, G.J. (1980) *Comp. Biochem. Physiol. 65B,* 207-214.
30. Gillan, F.T. (1983) *J. Chromatogr. Sci. 21,* 293-297.
31. Morales, R.W., and Litchfield, C. (1976) *Biochim. Biophys. Acta 431,* 206-216.
32. Litchfield, C., and Marcantonio, E.E. (1978) *Lipids 13,* 199-202.
33. Litchfield, C., Tyszkiewicz, J., and Dato, V. (1980) *Lipids 15,* 200-202.
34. Ayanoglu, E., Popov, S., Kornprobst, J.M., Aboud-Bichara, A., and Djerassi, C. (1983) *Lipids 18,* 830-836.
35. Ayanoglu, E., Kornprobst, J.M., Aboud-Bichara, A., and Djerassi, C. (1983) *Tetrahedron Lett.,* 1111-1114.
36. Lankelma, J., Ayanoglu, E., and Djerassi, C. (1983) *Lipids 18,* 853-858.
37. Wijekoon, W.M.D., Ayanoglu, E., Djerassi, C. (1984) *Tetrahedron Lett.,* 3285-3288.
38. Ayanoglu, E., Kurtz, K., Kornprobst, J.M., and Djerassi, C. (1985) *Lipids 20,* 141-144.
39. Lawson, M.P., Bergquist, P.R., and Cambie, R.C. (1986) *Tissue Cell 18,* 19-26.
40. Lawson, M.P., Thompson, J.E., and Djerassi, C. (1988) *Lipids 23,* 741-749.
41. Lawson, M.P., Stoilov, I.L., Thompson, J.E., and Djerassi, C. (1988) *Lipids 23,* 750-754.
42. Zimmerman, M.P., Thomas, F., Thompson, J.E., Djerassi, C., Streiner, H., Evans, E., and Murphy, P.T., *Lipids 24,* in press.
43. Kenyon, C.N. (1972) *J. Bacteriol. 109,* 827-834.
44. Kenyon, C.N., Rippka, R., and Stanier, R.Y. (1972) *Arch. Mikrobiol. 83,* 216-236.
45. Schneider, H., Gelpi, E., Bennett, E.O., and Oro, J. (1970) *Phytochemistry 9,* 613-617.
46. Duclaux, G. (1977) Ph.D. Thesis, Universite Pierre et Marie Curie, Paris.
47. Poirreck, M., and Pohl, P. (1984) *Phytochemistry 23,* 217-223.
48. Taylor, J., and Parkes, R.J. (1983) *J. Gen. Microbiol. 129,* 3303-3309.
49. Wilkinson, C.R., Garrone, R., and Vacelet, J. (1984) *Proc. R. Soc. London B220,* 519-528.
50. Litchfield, C., and Morales, R.W. (1976) in *Aspects of Sponge Biology* (Harrison, F.W., and Cowden, R.K., eds.), pp. 183-200, Academic Press, New York, NY.
51. Ha, T.B., Kokke, W.C.M.C., Thompson, J.E., Proudfoot, J., and C. Djerassi (1985) *Steroids 45,* 263-276.
52. Hofheinz, W., and Oesterhelt, G. (1979) *Helv. Chim. Acta 62,* 1307-1309.
53. Li, X., and Djerassi, C. (1983) *Tetrahedron Lett. 24,* 665-668.
54. Stoilov, I., Back, T.G., Thompson, J.E., and Djerassi, C. (1986) *Tetrahedron 42,* 4156-4162.
55. Carballeira, N.M., Maldonado, L., and Porras, B. (1987) *Lipids 22,* 767-769.
56. Carballeira, N.M., and Maldonado, L. (1986) *Lipids 21,* 470-471.

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METHODS

Rapid Separation of Neutral Lipids, Free Fatty Acids and Polar Lipids Using Prepacked Silica Sep-Pak Columns

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A method is described for the separation of neutral lipid, free fatty acid and polar lipid classes using small (600 mg), prepacked silica Sep-Pak columns. Combinations of hexane and methyltertiarybutylether were used to progressively elute cholesteryl ester first then triglyceride from the column. After column acidification, fatty acids were eluted followed by cholesterol. Recoveries of these lipids were 96% or greater. Polar lipids were eluted from the column using combinations of methyltertiarybutylether, methanol and ammonium acetate. Phospholipid classes could not be separated completely from each other. Phosphatidylethanolamine and phosphatidylinositol eluted together, whereas the more polar phosphatidylcholine, sphingomyelin and lysophosphatidylcholine were eluted as a second fraction. Recoveries of each phospholipid was greater than 98%.
Lipids 23, 1146-1149 (1988).

Methods to separate and isolate neutral and polar lipids have largely depended on preparative thin layer chromatography (TLC) and column chromatography using silicic acid, florisil or alumina and more recently on high performance liquid chromatography (HPLC) (1-4). Significant problems are associated with each of these methods. Preparative TLC is sensitive to sample overload, is cumbersome and time-consuming. Traditional column chromatography is time-consuming and requires large quantities of solvents. HPLC requires expensive equipment. Therefore, the need for rapid, efficient, high recovery methods are needed, especially for use with small and medium sample sizes.

In previous work, it was demonstrated that small silica Sep-Pak columns (600 mg) could be used to separate neutral and polar lipids with a greater than 98% recovery of the following lipid classes: cholesteryl esters, triglycerides, fatty acids, cholesterol, phosphatidylethanolamine and phosphatidylcholine (5). Others have reported the successful use of this and similar methods (6,7).

This report describes in detail the use of the disposable silica Sep-Pak columns to separate cholesteryl esters, cholesterol, free fatty acids and triglycerides from each other and from polar lipids using a novel solvent system containing methyltertiarybutylether (MTBE). The data were generated using radiolabeled and unlabeled lipid standards and lipids extracted from human serum or rat livers. Recoveries for each lipid class were excellent, as in the previous work (5). The processing of 10 samples can be accomplished in less than 1 hr. The use of MTBE instead of chloroform, which was used with the previous method, has allowed us to separate cholesteryl esters from triglycerides and to completely separate phosphatidylethanolamine from phosphatidylcholine.

MATERIALS AND METHODS

Lipids were extracted from human serum using the method of Bligh and Dyer (9). Extracts were stored under nitrogen at −20°C.

Radiolabeled rat liver phosphatidylcholine and phosphatidylethanolamine were prepared by intraperitoneal injection of either 5 mCi of [^{3}H]choline chloride or 5 mCi [^{14}C]ethanolamine (New England Nuclear, Boston, MA) into rats as described previously (5). After 24 hr, the rats were killed and the livers were extracted using the Bligh and Dyer method (8). The phospholipid fraction was obtained by separation from neutral lipids and fatty acids using a silica Sep-Pak column procedure (5). Radiolabeled sphingomyelin was prepared by a modification of the procedure of Dawson (9) using the lipids from the rat liver labeled with [^{3}H]choline chloride. Briefly, liver lipid was dissolved in 5 ml of 2% potassium hydroxide in 95% ethanol and heated at 37°C for 20 min. Chloroform (5 ml) was added to the mixture followed by 8 ml of distilled water. The upper water layer containing choline was discarded. The chloroform (lower) layer was washed twice with 55% methanol. The radioactivity contained in the chloroform layer was almost exclusively sphingomyelin (9). A small amount of additional radioactivity was removed by treating the sample with hydrochloric acid under mild conditions, indicating that plasmalogens were minimally labeled (9).

Neutral lipid standards were purchased from NuChek Prep (Elysian, MN) and stored in sealed vials at −20°C. Polar lipid standards were purchased from Avanti Polar Lipids (Birmingham, AL) and stored in sealed vials at −20°C. All solvents were HPLC grade and were purchased from Burdick and Jackson (Muskegon, MI). Silica Sep-Pak columns (600 mg silica) were purchased from Waters Associates (Milford, MA). The column void volume is 1.5 ml and the column capacity is greater than 1 mg lipid. Tri[^{14}C]oleylglycerol, [^{3}H]oleic acid, cholesteryl[^{3}H]oleate, [^{14}C]cholesterol, [^{3}H]phosphatidylinositol and [^{14}C]lysophosphatidylcholine were purchased from New England Nuclear (Boston, MA). The radiolabeled lipids were purified by HPLC prior to recovery experiments (5). Radiolabeled counting was performed using a Searle Analytic Mark III Scintillation Counter. Samples were mixed with 10 ml Aquasol (New England Nuclear, Boston, MA). For quantitative recovery experiments, the following radiolabeled lipids were added to lipids extracted from human serum (equivalent to 100 μl human serum): 0.02 μCi of [^{3}H]cholesteryl oleate, 0.1 μCi of [^{14}C]cholesterol, 0.02 μCi of [^{3}H]oleic acid, 0.02 μCi of tri[1-^{14}C]oleyl glycerol, 0.04 μCi of [^{3}H]phosphatidylinositol and 0.02 μCi of [^{14}C]lysophosphatidylcholine. Addi-

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tionally, 0.04 μCi of [^{14}C]phosphatidylethanolamine, 0.04 μCi of [^{3}H]phosphatidylcholine and 0.02 μCi [^{3}H]sphingomyelin were used without dilution as isolated from rat liver lipids. The column separations described below were performed three to six times for each individual radiolabeled lipid added to serum lipids and for combined radiolabeled lipids in serum lipids.

RESULTS

Separation of neutral lipids. Separation of neutral lipids and free fatty acids from polar lipids was accomplished using prepacked silica Sep-Pak columns as described previously (5), but replacing chloroform with MTBE in the elution solvent. Prior to use, each Sep-Pak column was washed with 4 ml of hexane/MTBE (96:4) followed by 12 ml hexane. This washing procedure removed substances that interfere with HPLC procedures used to monitor lipids (5,10).

Serum lipids from the Bligh and Dyer extraction equivalent to 100 μl of human serum were evaporated to dryness under nitrogen, dissolved in 2.0 ml of hexane/MTBE (200:3) and applied to the Sep-Pak column. The vessel containing the lipid extract was washed once with 2 ml hexane/MTBE (200:3) and the solvent added to the column. The 2 ml eluted solvent was saved.

Cholesteryl esters and triglycerides were eluted with combinations of hexane/MTBE. Hexane/MTBE (200:3, 10 ml) was added to the column, which resulted in the elution of cholesteryl esters in a total volume of 12 ml. The recovery of cholesteryl ester based on [^{3}H]cholesteryl oleate was 95.9% (Table 1). Using 12 ml of hexane/MTBE (96:4), the triglycerides emerged. The recovery of triglyceride based on added tri[1-^{14}C]oleylglycerol was 99% (Table 1).

After removal of triglycerides, the column was acidified with 12 ml hexane/acetic acid (100:0.2). This fraction contained no lipid and was discarded. Fatty acids were then eluted using 12 ml of hexane/MTBE/acetic acid (100:2:0.2) with a 98.2% recovery based on added [^{3}H]oleic acid (Table 1). Cholesterol was eluted using 12 ml of MTBE/acetic acid (100:0.2). Recovery based on added [^{14}C]-cholesterol was 99.1% (Table 1).

Separation of polar lipids. After the neutral lipids and fatty acids were eluted, polar lipids were eluted from the column using combinations of MTBE/methanol/ammonium acetate (pH 8.6). The aqueous ammonium acetate was prepared by adding 2 volumes of 0.001 M ammonium hydroxide to 1 volume of 0.001 M acetic acid. Approximately 50% of the phosphatidylinositol was eluted with MTBE/methanol/ammonium acetate (pH 8.6) (25:4:1, 8 ml) and the remaining 50% with MTBE/methanol/ammonium acetate (pH 8.6) (10:4:1, 12 ml) for a total recovery of 98.6% (Table 2). Phosphatidylethanolamine was quantitatively eluted in the 12 ml of MTBE/methanol/ammonium acetate (pH 8.6) (10:4:1) with a 98.7% recovery. Using 12 ml MTBE/methanol/ammonium acetate (pH 8.6) (5:4:1), 69% of the phosphatidylcholine, 50% sphingomyelin and 2% lysophosphatidylcholine were eluted (Table 2). By increasing the polarity of the eluting solvent to MTBE/methanol/ammonium acetate (pH 8.6) (5:8:2), an additional 22% phosphatidylcholine, 50% sphingomyelin and 93.3% lysophosphatidylcholine eluted (Table 2). An additional 12 ml of this solvent resulted in minor additional recoveries of lysophosphatidylcholine (Table 2). Overall recoveries were 91.6% for phosphatidylcholine, 100% for sphingomyelin and 99.9% for lysophosphatidylcholine based on recoveries of the radiolabeled standards.

Bulk separation of neutral and polar lipids. Bulk separation of all neutral lipids and fatty acids from polar lipids was accomplished with the Sep-Pak column using MTBE/acetic acid (100:0.2) as the eluting solvent. Lipids were dissolved in 2.0 ml of MTBE/acetic acid (100:0.2), applied to the column and washed once with an additional 2.0 ml of the solvent. The elution of all (98+%) neutral lipids and fatty acids was achieved with an additional 10 ml of the solvent. No polar lipdis were removed with MTBE/acetic acid (100:0.2). The polar lipids were then eluted using the solvent systems described above with recoveries identical to those in Table 2. The polar lipids were quantitatively separated into two fractions. The less polar lipids, e.g., phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidylglycerol, were eluted with MTBE/methanol/ammonium acetate (pH 8.6) (10:4:1) followed by the elution of more polar

TABLE 1

Separation and Percent Recovery of Neutral Lipids and Fatty Acids on Silica Sep-Pak Columns[a]

Lipid class	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Cholesteryl ester	95.9 ± 0.7	0	0	0
Triglyceride	0	99.0 ± 0.2	0	0
Fatty acid	0	0	98.2 ± 0.7	0
Cholesterol (and partial glycerides)	0	0	0	99.1 ± 0.3
Phosphatidylethanolamine	0	0	0	0
Phosphatidylcholine	0	0	0	0

[a]Lipids were extracted from fasted human serum using the method of Bligh and Dyer (8). Radiolabeled lipid standards purified by HPLC (5) were added separately to an equivalent of 100 μl of human serum lipid to determine recovery. Results are expressed as the percentage mean ± SE for a minimum of 4 separate determinations of each lipid class. Combinations of lipids were also separated with the same recoveries. Fraction 1 solvent is hexane/MTBE (200:3, 12 ml); fraction 2 solvent is hexane/MTBE (96:4, 12 ml); fraction 3 solvent is hexane/MTBE/acetic acid (100:2:0.2, 12 ml); fraction 4 solvent is MTBE/acetic acid (100:0.2, 12 ml).

TABLE 2

Separation and Percent Recovery of Polar Lipids on Silica Sep-Pak Columns[a]

Lipid class	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Phosphatidylethanolamine	0	98.7 ± 1.5	0	0	0
Phosphatidylinositol	51.5 ± 0.8	47.1 ± 1.0	0	0	0
Phosphatidylcholine	0	0	69.7 ± 1.1	21.9 ± 0.8	<0.1
Sphingomyelin	0	0	50.1 ± 1.4	49.8 ± 1.3	<0.1
Lysophosphatidylcholine	0	0	2.4 ± 1.0	93.3 ± 0.9	4.2 ± 1.0

[a]Lipids were extracted from fasted human serum using the method of Bligh and Dyer (8). Radiolabeled lipid standards purified by HPLC (5) were added separately to an equivalent of 100 μl of human serum lipid to determine recovery. Results are expressed as the percentage mean ± SE for a minimum of 4 separate determinations for each lipid class. Combinations of lipids were also separated with the same recoveries. The Sep-Pak column was washed with 12 ml MTBE/acetic acid (100:0.2), no radioactivity emerged. The solvent system was composed of MTBE/methanol/ammonium acetate (pH 8.6) in the following proportions and amounts: fraction 1, 25:4:1, 8 ml; fraction 2, 10:4:1, 12 ml; fraction 3, 5:4:1, 12 ml; and fraction 4 and 5, 5:8:2, 12 ml.

lipids, e.g., phosphatidylcholine, sphingomyelin and lysophosphatidylcholine, with MTBE/methanol/ammonium acetate (pH 8.6) (5:8:2). All phospholipids could be eluted with 10 to 20 ml of the latter solvent. Using this solvent is better than methanol alone because the phospholipids emerge in a minimum of solvent.

DISCUSSION

We have developed a rapid highly reproducible method to separate the major neutral lipid classes and polar lipids. By including the appropriate internal lipid standards prior to separation, excellent quantitative data was obtained. Radiolabeled lipids were used in the present work. However, quantitation can be achieved using chemical determination, gas chromatography or HPLC. The method described in this report can be used as an alternative to thin layer chromatography.

The separations obtained on the small disposable silica Sep-Pak columns were a function of lipid polarity and of solvent strength and polarity. The solvent proportions determined empirically were critical for complete separations. The elution volumes could be varied from 10 to 25 ml without contamination from the more polar lipids. A compromise volume of 12 ml was sufficient for complete elution of each lipid in the current work. The neutral lipids (cholesteryl esters and triglycerides) were eluted by solvent strength using combinations of hexane/MTBE; 200:3 for cholesteryl esters followed by 96:4 for triglycerides. A combination of 200 μg of cholesteryl ester and 100 μg of triglyceride separated readily. If present, methylesters of fatty acids and simple alkyl esters would not separate from cholesteryl esters and would elute with hexane/MTBE (200:3). Both short and long chain triglycerides elute with hexane/MTBE (96:4). After acidification of the column with hexane/acetic acid (100:0.2), fatty acids were eluted using hexane/MTBE/acetic acid (100:2:0.2). The elution of cholesterol was achieved with MTBE-acetic acid 100:0.2. Monoglycerides, 1,2- and 1,3-diglycerides emerged in the cholesterol fraction as measured by lipid standards and HPLC chromatography, but they were not quantitated using radiolabeled standards for this report.

Phospholipid classes could not be separated completely from each other. However, phosphatidylethanolamine and phosphatidylinositol were separated completely from phosphatidylcholine, sphingomyelin and lysophosphatidylcholine with excellent (98+%) recoveries. Separations of phosphatidylethanolamine from phosphatidylinositol or phosphatidylcholine from sphingomyelin and lysophosphatidylcholine were not achieved. However, after partial separation on the silica Sep-Pak column, these polar lipids can be separated by HPLC with excellent recovery (10).

This rapid, simple method lends itself to enzymic and other assays where measurement of a lipid as an endpoint is necessary. It is particularly useful for radiolabeled methods. Incorporation of cholesterol precursor into cholesterol, incorporation of fatty acid into cholesterol ester where separation of cholesterol, cholesteryl ester and fatty acid is desired and the separation of fatty acids and triglycerides during lipolysis are a few examples of potential applications of this methodology. Additionally, the cartridge can be reused if washed with methanol (20 ml), MTBE (20 ml) and then hexane (20 ml) making the separations more economical.

In addition to human serum lipids, rat serum and liver lipids, as well as lipids from cultured smooth muscle cells, have been separated quantitatively, as in previous work (5).

The most difficult separation of the neutral lipids proved to be the separation of cholesteryl esters from triglycerides. Numerous combinations of hexane and more polar solvent mixtures were tried. MTBE and diethylether proved to be better than others such as ethyl acetate, chloroform or dichloromethane. MTBE was preferred to diethylether because diethylether is much less stable than MTBE and many of the stabilizers interfered with the analysis. It was surprising to find that the separation of phosphatidylethanolamine from phosphatidylcholine was better using MTBE than chloroform.

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REFERENCES

1. Marinetti, G. (ed.) (1982) Vol. 1, Marcel Dekker Inc., New York, NY.
2. Christie, W.W. (1982) *Lipid Analysis,* 2nd edn., pp. 96–98, Pergamon Press, New York, NY.
3. Aitzemüller, K. (1982) *Prog. Lipid Res. 21,* 171–193.
4. Carroll, R.M., and Rudel, L.L. (1981) *J. Lipid Res. 22,* 359–363.
5. Hamilton, J.G., and Comai, K. (1984) *J. Lipid Res. 25,* 1142–1148.
6. Kaluzny, M.A., Duncan, L.A., Merritt, M.V., and Epps, D.E. (1985) *J. Lipid Res. 26,* 135–140.
7. Figewicz, D.A., Nolon, C.E., Singh, I.N., and Jungalwala, F.B. (1985) *J. Lipid Res. 26,* 140–144.
8. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911–917.
9. Dawson, R.M.C. (1960) *Biochem. J. 75,* 45–53.
10. Hamilton, J., and Comai, K. (1988) *Lipids 23,* 1150–1153.

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Separation of Neutral Lipid, Free Fatty Acid and Phospholipid Classes by Normal Phase HPLC

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Normal phase high performance liquid chromatography methods are described for the separation of neutral lipid, fatty acid and five phospholipid classes using spectrophotometric detection at 206 nm. Separations were accomplished in less than 10 min for each lipid class. A mobile phase consisting of hexane/methyltertiarybutylether/acetic acid (100:5:0.02) proved effective in separating cholesteryl ester and triglyceride with recoveries of 100% for radiolabeled cholesteryl oleate and 98% for radiolabeled triolein. Free fatty acid and cholesterol were separated by two different mobile phases. The first, hexane/methyltertiarybutylether/acetic acid (70:30:0.02) effectively separated free fatty acids and cholesterol, but did not separate cholesterol from 1,2-diglyceride. A mobile phase consisting of hexane/isopropanol/acetic acid (100:2:0.02) effectively separated free fatty acid, cholesterol, 1,2-diglyceride and 1,3-diglyceride. Recoveries of oleic acid and cholesterol were 100% and 97%, respectively. Five phospholipid classes were separated using methyltertiarybutylether/methanol/aqueous ammonium acetate (pH 8.6) (5:8:2) as the mobile phase. The recoveries of phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin and lysophosphatidylcholine were each greater than 96%.
Lipids 23, 1150–1153 (1988).

The development of mobile phases for separations of lipids using high performance liquid chromatography (HPLC) has improved despite the lack of adequate detection techniques (1–3). Mobile phases have been developed so that the ultraviolet absorbing properties of lipids in the range of 190 to 210 nm can be used to monitor separations (2–4). Solvents such as methyltertiarybutylether (MTBE), hexane, isopropanol, acetonitrile and methanol, which do not absorb appreciably in the ultraviolet range of 190 to 210 nm, have been used successfully in both normal and reversed phase HPLC separations of lipids (2–5).

This report describes in detail normal phase HPLC techniques for quantitative separation of neutral lipid classes, fatty acids and five major phospholipid classes. Separations were complete for all lipids with the exception of a 15% cross contamination of phosphatidylcholine and sphingomyelin. Each isocratic HPLC separation is completed in less than 10 min with recoveries of greater than 96% for each lipid.

MATERIALS AND METHODS

Lipids were extracted from human serum and rat liver using the method of Bligh and Dyer (6). Extracts were stored under nitrogen at −20°C. Radiolabeled rat liver [^{3}H]phosphatidylcholine, [^{14}C]phosphatidylethanolamine and [^{3}H]sphingomyelin were prepared as previously described (4,7,8). Tri[1-^{14}C]oleylglycerol, [^{3}H]oleic acid, cholesteryl [^{3}H]oleate, [^{14}C]cholesterol, [^{3}H]phosphatidylinositol and [^{14}C]lysophosphatidylcholine were purchased from New England Nuclear (Boston, MA). These radiolabeled lipids were purified by HPLC prior to performing recovery experiments (4). For quantitative recovery experiments, the following amounts of radiolabeled lipids were used: 0.02 μCi of [^{3}H]cholesteryl oleate, 0.1 μCi of [^{14}C]cholesterol, 0.02 μCi of [^{3}H]oleic acid, 0.02 μCi of tri[1-^{14}C]oleylglycerol, 0.04 μCi of [^{3}H]phosphatidylinositol and 0.02 μCi of [^{14}C]lysophosphatidylcholine. Additionally, 0.04 μCi of [^{14}C]phosphatidylethanolamine, 0.04 μCi of [^{3}H]phosphatidylcholine and 0.02 μCi of [^{3}H]-sphingomyelin were used without dilution as isolated from rat liver lipids. Radiolabeled counting was performed using a Searle Analytic Mark III Scintillation Counter. Samples for counting were suspended in 10 ml Aquasol (New England Nuclear, Boston, MA).

Neutral lipid standards were purchased from NuChek Prep (Elysian, MN) and stored in sealed vials at −20°C. Polar lipid standards were purchased from Avanti Polar Lipids (Birmingham, AL) and stored in sealed vials at −20°C. All solvents were HPLC grade and were purchased from Burdick and Jackson (Muskegon, MI).

HPLC was performed using a Waters Associates (Milford, MA) Model 840 HPLC work station which includes a Digital Professional 350 Computer, a Digital LA 50 printer, a Waters 481 Spectrophotometer operated at 206 nm, two Waters Associates model 510 pumps and a Waters Associates U6K injector. Prepacked μPorasil columns (30 cm × 3.9 mm) were obtained from Waters Associates (Milford, MA). As much as 1 mg of each lipid standard was used without effect on column performance. The column capacity for lipid mixtures is greater than 2 mg. The mobile phase flow rate was 2.0 ml/min. All injections for quantitative measurements were made with a TP#5250 syringe (Unimetrics Corporation, Anaheim, CA). All samples for HPLC separations were dissolved in the solvent that was used as the mobile phase. The HPLC system was maintained at room temperature, ca. 22°C.

RESULTS

Separation of neutral lipid classes and free fatty acids by HPLC. Several mobile phases were developed to separate neutral lipids and free fatty acids by normal phase HPLC.

Figure 1 shows the HPLC chromatogram of serum lipids containing [^{3}H]cholesteryl oleate and tri[1-^{14}C]-oleylglycerol standards. The mobile phase consisting of hexane/MTBE/acetic acid (100:5:0.02) (mobile phase 1)

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Abbreviations: Ch, cholesterol; CO, cholesteryl oleate; 1,2DG, 1,2-dioleylglycerol; 1,3DG, 1,3-dioleylglycerol; LPC, lysophosphatidylcholine; MTBE, methyltertiarybutylether; OA, oleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SP, sphingomyelin; TO, triolein; HPLC, high performance liquid chromatography.

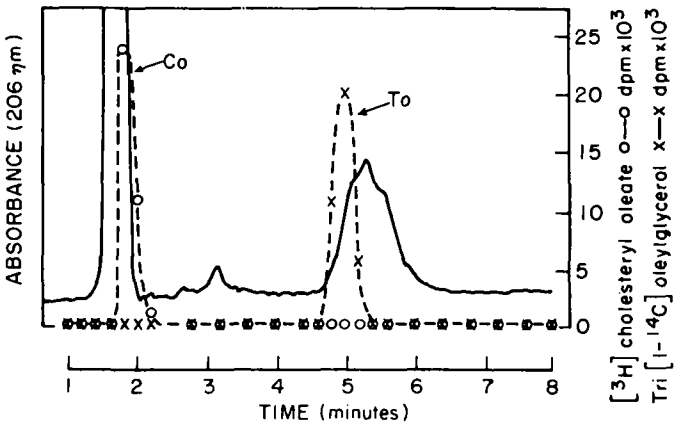

FIG. 1. HPLC separation of serum lipids equivalent to 100 μl serum containing [^{3}H]cholesteryl oleate and tri[1-^{14}C]oleylglycerol. Column: 30 cm × 3.9 mm μPorasil; flow rate: 2.0 ml/min; detection 0.05 AUFS at 206 nm; mobile phase (1): hexane/MTBE/acetic acid (100:5:0.02). [^{3}H]cholesteryl oleate (O———O). Tri[1-^{14}C]oleylglycerol (X———X).

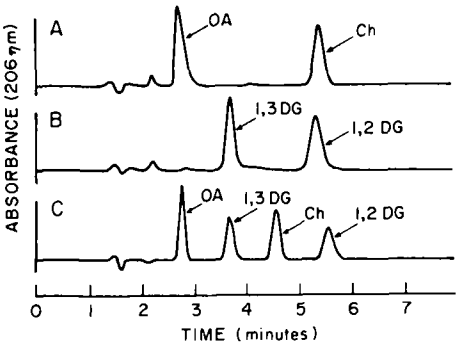

FIG. 2. HPLC separation of neutral lipids. Column: 30 cm × 3.9 mm μPorasil; flow rate: 2.0 ml/min. A and B: detection 0.05 AUFS at 206 nm; mobile phase, hexane/MTBE/acetic acid (70:30:0.02). C: detection 0.15 AUFS at 206 nm; mobile phase, hexane/isopropanol/acetic acid (100:2:0.02). Peak identity: OA, oleic acid, 50 μg; 1,3DG, 1,3-dioleylglycerol, 20 μg; Ch, cholesterol, 5 μg; 1,2DG, 1,2-dioleylglycerol, 20 μ.

effectively separated cholesteryl esters from triglycerides. The radiolabeled standards emerged as sharp peaks at 2.0 min for [^{3}H]cholesteryl oleate and 5.2 min for tri[1-^{14}C]oleylglycerol with excellent recoveries of each (Table 1). However, mobile phase 1 was not sufficiently polar to separate cholesterol and free fatty acids in a reasonable period of time (less than 20 min). Increasing the flow rate of mobile phase 1 to 4.0 ml/min did not appreciably improve the chromatographic profile. The polarity of mobile phase 1 was increased by the addition of more MTBE. The HPLC chromatogram of cholesterol and free fatty acids using hexane/MTBE/acetic acid (70:30:0.02) (mobile phase 2) is shown in Figure 2A. Fatty acids and cholesterol were clearly separated from each other within 7 min. The radiolabeled oleic acid standard emerged at 2.7 min with a 95.9% recovery and the [^{14}C]-cholesterol standard emerged at 5.8 min with a recovery of 98.9% (Table 1). Using mobile phase 2, another neutral lipid, 1,3-dioleylglycerol, separated from both oleic acid and cholesterol (Fig. 2B). However, 1,2-dioleylglycerol did not separate from cholesterol (Fig. 2B). Consequently, a third mobile phase consisting of hexane/isopropanol/acetic acid (100:2:0.02) was developed which effectively separated oleic acid, 1,3-dioleylglycerol, cholesterol and 1,2-dioleylglycerol (Fig. 2C). Oleic acid emerged at 2.7 min, 1,3-dioleylglycerol emerged at 3.7 min, cholesterol at 4.6 min and 1,2-dioleylglycerol at 5.6 min. The recoveries of oleic acid and cholesterol based on the added radiolabeled standards was greater than 98% for each lipid (Table 1). Radiolabeled 1,2- and 1,3-dioleylglycerols were not available and thus recoveries were not determined.

Separation and recoveries of phospholipid classes by HPLC. Phospholipid standards were chromatographed separately and in combination as shown in Figure 3. The solvent system that had been successful in separating phospholipids on small silica Sep-Pak columns (4) was used as the mobile phase for the HPLC separations. This mobile phase consisted of MTBE/methanol/ammonium acetate (pH 8.6) (5:8:2) (4). The ammonium acetate was prepared by adding 2 volumes of 0.001 M ammonium hydroxide to 1 volume of 0.001 M acetic acid.

Within 10 min, the separation of the five phospholipid

TABLE 1

Recovery of Neutral Lipids and Free Fatty Acids from HPLC

Lipid class[a]	% Recovery		
	Mobile phase 1[b]	Mobile phase 2[c]	Mobile phase 3[d]
Cholesteryl ester	100.2 ± 0.6	—	96.9 ± 1.0
Triglyceride	98.2 ± 0.7	—	97.8 ± 0.7
Free fatty acid	—	95.9 ± 0.3	100.0 ± 0.5
Cholesterol	—	98.9 ± 0.7	97.4 ± 1.4

[a]Lipids were extracted from human serum using the method of Bligh and Dyer (6). Radiolabeled lipid standards were added separately to an equivalent of 100 μl of human serum lipid. Radiolabeled lipid standards were also added in combination.

[b]Mobile phase 1: hexane/MTBE/acetic acid (100:5:0.02). The results are the mean ± SE for 5 determinations of each lipid class.

[c]Mobile phase 2: hexane/MTBE/acetic acid (70:3:0.02). The results are the mean ± SE for 6 determinations of each lipid class.

[d]Mobile phase 3: hexane/isopropanol/acetic acid (100:2:0.02). The results are the mean ± SE for 7 determinations for each lipid class.

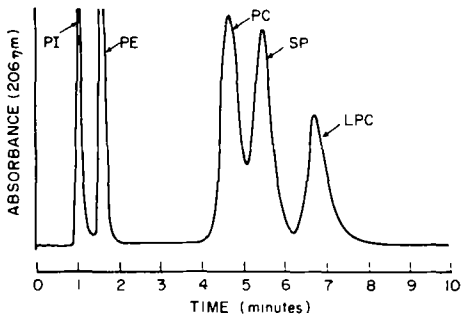

FIG. 3. Separation of phospholipid classes. Column: 30 cm × 3.9 mm μPorasil; flow rate: 2.0 ml/min; detection 0.3 AUFS at 206 nm. Mobile phase (4): MTBE/methanol/ammonium acetate (pH 8.6) (5:8:2). Peak identity: PI, phosphatidylinositol (liver), 25 μg; PE, phosphatidylethanolamine (liver), 125 μg; PC, phosphatidylcholine (egg), 100 μg; SP, sphingomyelin (egg), 100 μg; LPC, monoleyllysophosphatidylcholine (synthetic), 400 μg.

classes was complete (Fig. 3), with recoveries of greater than 06% for each (Table 2).

TABLE 2

Recovery of Phospholipids from HPLC

Lipid[a]	% Recovery[b]
Phosphatidylinositol	98.7 ± 0.9
Phosphatidylethanolamine	98.3 ± 1.1
Phosphatidylcholine	96.1 ± 0.9
Sphingomyelin	100.0 ± 0.8
Lysophosphatidylcholine	96.5 ± 0.2

[a]Radiolabeled lipid standards were combined with unlabeled lipid standards: 25 μg unlabeled + 0.04 μCi of phosphatidylinositol; 125 μg unlabeled + 0.04 μCi of [^{14}C]phosphatidylethanolamine; 100 μg unlabeled + 0.04 μCi of [^{3}H]phosphatidylcholine; 100 μg unlabeled + 0.02 μCi of [^{3}H]sphingomyelin; 400 μg unlabeled + 0.02 μCi of [^{14}C]lysomonoleylphosphatidylcholine. For each individual phospholipid and for the combination, the solvent was evaporated to dryness under nitrogen and taken up in 100 μl of the HPLC mobile phase; MTBE/methanol/aqueous ammonium acetate (pH 8.6) (5:8:2).

[b]The results are the mean ± SE for a minimum of 5 determinations for each individual phospholipid. The recoveries from independent determinations were similar to recoveries from mixtures of these phospholipids.

DISCUSSION

Although there are several very good reversed phase HPLC methods for the separation of the individual species of cholesteryl esters and triglycerides (2,9), few HPLC methods are available for the separation and quantitative recovery of the classes of cholesteryl esters and triglycerides (4,10). The method currently reported is substantially improved over the previously published method in that the silica column step can be omitted and the separation of cholesteryl ester from triglyceride is complete (4). An important aspect of this method is that the elution schemes are isocratic and thus require a single pump rather than a costly gradient system. Additionally, the mobile phases were selected to give complete separations in short periods of time. The cholesteryl ester fraction of serum emerged at 1.4 to 2.0 min, when hexane/MTBE/acetic acid (100:5:0.02) was used as the mobile phase (Fig. 1). Serum triglycerides emerged between 4.8 and 6.0 min with this procedure (Fig. 1). The radiolabeled cholesteryl oleate and triolein used as standards, emerged as sharp peaks when plotted on the HPLC chromatogram. The recoveries were excellent, 100% for cholesteryl oleate and 98% for triolein (Table 1). This method allows for an easy, rapid and quantitative separation of cholesteryl esters from triglycerides. The volumes of solvents used were not critical, However, the proportions of solvents were key to complete separation of cholesteryl ester from triglyceride. Once these lipid classes have been separated, separation into their respective molecular species can be achieved by reversed phase HPLC methods (1).

A second mobile phase, hexane/MTBE/acetic acid (70:30:0.02), effectively separated free fatty acids and cholesterol with quantitative recovery of each (Fig. 2A, Table 2). However, if 1,2-diglycerides are present in the lipid mixture, they will not be separated from cholesterol (Figs. 2A and B). A third mobile phase consisting of hexane/isopropanol/acetic acid (100:2.0:0.02) was used to effectively separate fatty acids, 1,3-diglycerides, cholesterol and 1,2-diglycerides. With this solvent system, carbon chain length and double bonds have little influence on separation, i.e., all fatty acids emerge as a single peak and are separated from alcohols which also emerge as a single peak (Fig. 2C) (4). This mobile phase has the advantage of collecting several individual lipid classes from a single chromatographic run. It has the disadvantage that cholesteryl esters and triglycerides emerge (quantitatively) in the void volume (Table 1).

A number of excellent methods exist for the separation of phospholipid classes using HPLC (3,5,10). The advantage of the current method is that the separation is completed in less than 10 min compared to the 60 to 120 min in most other published work (3,5,10). There is a 15% cross contamination of sphingomyelin and phosphatidylcholine. However, an excellent (98+%) recovery of each phospholipid class is achieved. Although not shown in Figure 3, phosphatidylglycerol and cardiolipin emerge in the phosphatidylinositol fraction.

We have used a combination of the above procedures to separate lipids from serum, liver and cultured cells. It has become routine to use prepacked silica columns to separate neutral lipids and fatty acids from polar lipids (7) and then to use mobile phases 2 and 3 to separate and collect the neutral lipids and mobile phase 4 to separate and collect the phospholipids of interest.

It should be of interest to the potential user of these methods, that the HPLC columns remain stable at the pH of the solvent systems used. To reequilibrate the column after collection of the phospholipids, 3 to 5 column volumes of MTB/acetic acid (100:0.2) were used, followed by 10 to 15 column volumes of the neutral lipid solvent such as hexane/MTBE/acetic acid (100:5:0.02). The HPLC columns were good for at least 50 separations.

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REFERENCES

1. Aitzemüller, K. (1982) *Prog. Lipid Res. 21,* 171-193.
2. Carroll, R.M., and Rudel, L.L. (1981) *J. Lipid Res. 22,* 359-363.
3. Patton, G.M., Fasulo, J.M., and Robins, S.J. (1983) *J. Lipid Res. 23,* 190-196.
4. Hamilton, J.G., and Comai, K. (1984) *J. Lipid Res. 25,* 1142-1148.
5. Kaduce, T.L., Norton, K.C., and Spector, A.A. (1983) *J. Lipid Res. 24,* 1398-1403.
6. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37,* 911-917.
7. Hamilton, J.G., and Comai, K. (1988) *Lipids 23,* 1146-1149.
8. Dawson, R.M.C. (1960) *Biochem. J. 75,* 45-53.
9. Greenspan, M.D., and Schroeder, E.A. (1982) *Anal. Biochem. 127,* 441-448.
10. Schlager, S.I., and Jordi, H. (1981) *Biochim. Biophys. Acta 665,* 355-358.

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Erythrocyte Contamination of Leukocyte Populations Following Density-Gradient Centrifugation Results in Artificially High Levels of Human Leukocyte HMG-CoA Reductase Activity

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When measuring human leukocyte HMG-CoA reductase activity, special care must be taken to prevent erythrocyte contamination of the leukocyte layer during isopycnic centrifugation. Contamination during leukocyte isolation and subsequent erythrocyte lysis during NH_4Cl treatment results in increased leukocyte microsomal HMG-CoA reductase activity. Increased enzyme activity is not due to enzyme dephosphorylation, thiol-disulfide reduction or increased enzyme protein concentration. Leukocyte populations containing granulocytes appear to be most sensitive. Prevention of erythrocyte contamination during isopycnic centrifugation should aid in accurate measurement of human leukocyte HMG-CoA reductase activity.
Lipids 23, 1154–1158 (1988).

Measurement of human leukocyte HMG-CoA reductase (reductase) has been complicated by the low activity of the enzyme and the large number of leukocytes required for direct measurement of enzyme activity. This has led many investigators to rely on rates of conversion of acetate into cholesterol as an indirect measure of enzyme activity (1–6). Estimation of rates of cholesterol synthesis requires a smaller number of leukocytes than direct measurement of enzymatic activity, but does not always accurately reflect reductase activity. For example, in mononuclear leukocytes, only about 50% of the acetate converted into mevalonate is converted into cholesterol (7,8). Furthermore, granulocytes are unable to synthesize sterols, due to the absence of squalene epoxidase (2,9), and thus convert all mevalonate formed into nonsterol products.

Direct measurement of leukocyte reductase activity in mitochondria-free whole cell extracts (7,10–12) provides a better estimate of enzyme activity than acetate conversion to sterols (12). However, such extracts contain high concentrations of HMG-CoA cleavage enzymes that preclude accurate measurement of reductase activity by producing nonmevalonate products that comigrate with mevalonate during thin layer chromatography (12). We recently described methods for isolating human leukocyte microsomes and for measuring leukocyte microsomal reductase activity (8) that eliminate the HMG-CoA cleavage activity present in whole cell extracts (8) and thus afford a more accurate determination of enzyme activity than measurement in whole cell extracts (8,13–17).

All three methods described above for estimating human leukocyte HMG-CoA reductase activity, however, rely on density gradient centrifugation methods for separation of leukocytes from plasma and erythrocytes. We recently discovered that when this separation is poor, and erythrocytes contaminating the leukocyte population are lysed during subsequent NH_4Cl treatment, artifactually high levels of reductase activity result.

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Abbreviation: BSS, Balanced Salts Solution.

MATERIALS AND METHODS

Chemicals. Escherichia coli alkaline phosphatase was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were from previously listed sources (8,13,14, 17,18). Monospecific, polyclonal, rabbit, antirat liver reductase antiserum was a generous gift from Dr. G. C. Ness (University of South Florida, Tampa, FL).

Isolation of peripheral blood leukocytes. Blood samples ranging from 40 ml to 120 ml were obtained using tourniquet pressure from an antecubital vein with a 19-ga needle and 60-ml syringes. After removing the needle from the syringe, the blood was discharged into 50-ml conical plastic centrifuge tubes containing 1.0 ml of 250 mM EDTA (pH = 7.4) and the tubes were inverted several times. Care was taken to empty the syringe over 5 to 10 sec, with a jet of blood coursing along the side of the tube to minimize hemolysis. Within 10 min of blood drawing, the sample was diluted with an equal volume of room temperature Hank's Balanced Salts Solution (BSS). For isolation of mononuclear leukocytes, 20 ml aliquots of the diluted blood sample were immediately added to 50 ml plastic conical centrifuge tubes, underlayered with 20 ml of room temperature Histopaque 1077 and centrifuged for 40 min at 400 × *g*. To isolate total leukocyte populations (mononuclear leukocytes plus granulocytes), 20-ml aliquots of the diluted blood sample were underlayered with 20 ml of room temperature 70% isoosmotic Percoll (70 ml Percoll, 0.62 g NaCl and 30 ml 0.15 M NaCl) and centrifuged at 400 × *g* for 40 min. Isolated leukocyte populations were washed twice in Hank's BSS and then treated with 0.87% NH_4Cl for 10 min to lyse contaminating erythrocytes. The erythrocyte lysate was then removed from the leukocytes by centrifugation at 400 × *g* for 15 min. Isolated leukocyte populations were then washed and stored frozen in liquid N_2 as previously described (8). Further fractionation of total leukocyte populations into mononuclear leukocyte and granulocyte fractions was accomplished using an isoosmotic Percoll gradient, as described by Harwood et al. (8).

Isolation of plasma-free erythrocytes. Two additional ml of blood from samples used for leukocyte isolation were centrifuged for 5 min in a clinical centrifuge. Following centrifugation, the plasma and buffy coat were discarded. The erythrocyte pellet was washed twice in 4.0 ml Hank's BSS and resuspended to a final volume of 2.0 ml with Hank's BSS. Microsomal reductase activity of this fraction was negligible (data not shown).

Measurement of leukocyte microsomal reductase activity. Leukocyte microsomes were isolated and reductase activity was measured, as described by Harwood et al.

METHODS

(8). Frozen cell suspensions were incubated at room temperature until just thawed, diluted to 1.0 ml with fresh assay buffer (50 mM tris (pH = 7.5, 1.0 mM EDTA, 5.0 mM dithiothreitol, 50 mM KCl) and homogenized 15 times manually with a ground glass Potter-Elvehjem tissue homogenizer. Homogenates were next diluted to 3.0 ml with assay buffer and homogenized 10 times with a motor-driven Teflon pestle. The resulting homogenates were diluted to 8 ml with assay buffer and centrifuged, first at 2,000 × *g* to remove unbroken cells and cell debris, and then at 172,000 × *g* to sediment microsomes. Following centrifugation, the microsomal pellet was resuspended in assay buffer and assessed for reductase activity.

To assay leukocyte reductase activity, 150 μg leukocyte microsomal protein were incubated at 37°C for 30 min in a final volume of assay buffer containing 67 μM [3-^{14}C]HMG-CoA (specific activity 30 cpm/pmol), [5-^{3}H]-mevalonolactone (11,000 cpm; 629 Ci/mol) as an internal standard, 3.4 mM $NADP^+$, 30 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase and 68 mM EDTA to prevent conversion of mevalonate to phosphomevalonate during incubation. Following incubation, 10 μl of 6 M HCl were then added to terminate the reductase-catalyzed reaction and to convert newly formed mevalonate to mevalonolactone. The mevalonolactone was then separated from unreacted substrate by silica gel thin layer chromatography. Following development in toluene-acetone 1:1 (v/v), the region of the chromatogram from R_f 0.5 to R_f 1.0 was removed and counted in 4.0 ml of Scintelene liquid scintillation fluid.

Radioactivity contained in this region of the silica gel chromatogram comigrated in a 1:1 relationship with authentic [^{3}H]mevalonolactone on three separate thin layer chromatographic systems and was converted into phosphomevalonates, following incubation with MgATP and rat liver mevalonate kinase (data not shown; for similar results using cultured human leukocyte microsomes, see Ref. 8). No other radiolabeled reaction products were detectable in this region of the chromatogram. Thus, as was previously shown using cultured human leukocyte microsomes (8), HMG-CoA cleavage activity is apparently absent from these microsomal preparations. Taken together, these data indicate that mevalonate, the product of the reductase-catalyzed reaction, is the only radiolabeled reaction product migrating in this region of the chromatogram.

Reductase activity is expressed as pmol of mevalonate formed per min of incubation at 37°C per mg microsomal protein. Generally, triplicate determinations of enzyme activity (150 μg of microsomal protein per measurement) could be made from a 40-ml blood sampling.

Measurement of reductase protein concentration. Leukocyte microsomal reductase protein concentration was measured in 1.5 μg of microsomal protein using a noncompetitive, solid-phase enzyme immunoassay recently described by Harwood et al. (18). Purified, proteolytically modified human liver reductase (>94% homogeneous, as judged by scanning transmission densitometry [18]) was used as the source of purified enzyme protein standard. Reductase protein concentration is expressed as μg of immunoreactive protein per mg microsomal protein.

Analysis of protein. Microsomal protein concentration was determined by the method of Bradford (19) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Mononuclear leukocyte reductase activity from 31 healthy, normocholesterolemic individuals averaged 10.3 ± 0.8 (SE) pmol/min/mg. This value was not significantly different from that measured in isolated lymphocytes (10.1 ± 0.8 pmol/min/mg; n = 8), monocytes (13.0 ± 1.8 pmol/min/mg; n = 7), granulocytes (8.8 ± 0.5 pmol/min/mg; n = 3) or total leukocyte populations (9.3 ± 0.9 pmol/min/mg; n = 2). However, total leukocyte populations, visibly contaminated with erythrocytes following density-gradient centrifugation, but uncontaminated following NH_4Cl treatment (lyses erythrocytes), possessed enzyme activities exceeding 120 pmol/min/mg (Table 1). In one individual (subject 2), leukocytes from one portion of a single blood draw were contaminated with erythrocytes following density-gradient centrifugation, whereas two other portions were uncontaminated. The contaminated sample, which was uncontaminated following NH_4Cl treatment, contained ca. 7 times the microsomal reductase activity of the uncontaminated leukocytes (Table 1). Increased reductase activity was not due to the presence of residual erythrocytes during leukocyte homogenization and microsomal isolation, however, because all erythrocytes were lysed during NH_4Cl treatment and the lysate was removed prior to leukocyte homogenization. Furthermore, because erythrocytes do not contain significant amounts of microsomal reductase activity (data not shown), increased leukocyte reductase activity was not due to contamination of leukocyte microsomal reductase with erythrocyte microsomal reductase.

Increased enzyme activity was not the result of elevated reductase protein concentration (Table 2) but was due to increased enzyme catalytic efficiency (Table 2). Increased catalytic efficiency, however, was not due to enzyme dephosphorylation or thiol-disulfide reduction (Table 3), because addition of *E. coli* alkaline phosphatase or 20 mM dithiothreitol to leukocyte microsomes from a leukocyte sample, not contaminated during density-

TABLE 1

Increased Leukocyte Reductase Activity With Erythrocyte Contamination

Subject	Reductase activity (pmol/min/mg)		Fold increase
	Uncontaminated leukocytes	Contaminated leukocytes	
1	5.9 ± 0.2	124 ± 6	21.0
2	19.3 ± 0.3	135 ± 21	7.0
3	—	135 ± 15	—

Total leukocyte populations were isolated from 40 ml of peripheral blood using 70% isoosmotic Percoll. Data are the average of triplicate determinations of reductase activity ± S.E. For subjects 1 and 3, data are for two separate blood samplings, one which was grossly contaminated with erythrocytes following density-gradient centrifugation, but free from contamination following NH_4Cl treatment (lyses erythrocytes), and one which was uncontaminated (normal intrasubject variation <10%; [8]). For subject 2, data are for a single blood sample in which one of three portions showed gross erythrocyte contamination following density-gradient centrifugation, but not following NH_4Cl treatment, whereas the other two showed no contamination.

gradient centrifugation, failed to produce enzyme levels comparable with those generated in a second portion of the same leukocyte sample, contaminated by erythrocytes during density-gradient centrifugation, but uncontaminated following NH_4Cl treatment (Table 3).

TABLE 2

Increased Leukocyte Reductase Activity is Due to Increased Enzyme Catalytic Efficiency

	Reductase activity (pmol/min/mg)	Reductase protein concentration (μg/mg)	Reductase catalytic efficiency (pmol/min/mg)
	(A)	(B)	(A/B)
Uncontaminated (a)	19.3 ± 0.3	61.7 ± 3.7	347
Contaminated (b)	135 ± 21	85.0 ± 1.5	1,590
b/a	7.0	1.4	4.6

Reductase activity and enzyme protein concentration of the microsomes isolated from the leukocyte populations of subjects 1-3 (Table 1) were measured, as previously described. The data obtained for the leukocyte populations of subject 2 are presented above. Data are the average of triplicate determinations ± SE. Reductase catalytic efficiency, calculated by dividing enzyme activity by enzyme protein concentration, is expressed as pmol/min/mg immunoreactive protein.

Similar increases in catalytic efficiency were observed for the contaminated samples from subjects 1 and 3 (4,560 and 2,250 pmol/min/mg immunoreactive protein, respectively). As previously reported (18,21), the catalytic efficiency obtained from 31 uncontaminated mononuclear leukocyte populations averaged 313 ± 34 pmol/min/mg, a value consistent with the catalytic efficiencies obtained for the uncontaminated total leukocyte populations isolated from subjects 1 and 2.

TABLE 3

Increased Leukocyte Catalytic Efficiency is Not Due to Dephosphorylation or Thiol-Disulfide Reduction

Additions	Reductase activity (pmol/min/mg)
None	19.3 ± 0.3
Alkaline phosphatase	23.5 ± 1.1
20 mM dithiothreitol	21.7 ± 0.5
Erythrocyte contamination	135 ± 21

Peripheral blood leukocyte microsomes (150 μg) isolated from leukocyte populations of subject 2 (Table 1) were incubated at 37°C for 20 min in 50 μl of either buffer (50 mM tris (pH = 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, 70 mM KCl), buffer containing 10 U *E. coli* alkaline phosphatase or buffer containing 20 mM dithiothreitol. Enzyme activity was subsequently measured in a second 30-min incubation at 37°C. Data are the average of triplicate determinations ± SE and are compared with the value obtained from a population of the same leukocytes contaminated with erythrocytes following density-gradient centrifugation, but not following NH_4Cl treatment.

As previously reported (21), following isolation, the mononuclear leukocyte enzyme is fully active with respect to dephosphorylation and thiol-disulfide reduction. For example, for a series of mononuclear leukocyte populations for which activity was measured in the presence and absence of either dithiothreitol of alkaline phosphatase, enzyme activity averaged 8.6 ± 1.2 pmol/min/mg microsomal protein in the absence of both agents and was 105% of control in the presence of dithiothreitol and 88% of control in the presence of alkaline phosphatase. Similar results (109% of control and 100% of control, respectively) were observed for total leukocyte populations.

To determine whether increased enzyme activity was due to the presence of and/or lysis of contaminating erythrocytes during NH_4Cl treatment, we subjected whole blood samples to repetitive treatment with 0.87% NH_4Cl until all erythrocytes were lysed. Total leukocyte populations isolated in this way had 6-16 times the reductase activity of either uncontaminated mononuclear leukocyte populations from the same blood sample or uncontaminated total leukocyte populations from alternate blood samples (Table 4). When total leukocyte populations, isolated by this method, were separated into mononuclear leukocyte and granulocyte fractions by isoosmotic Percoll gradient centrifugation, reductase activity in the granulocyte fraction (>90% granulocytes, as determined by Wright's staining) was found to be increased 3-13-fold, when compared with either uncontaminated mononuclear leukocyte populations from the same blood sample or uncontaminated granulocyte populations from alternate blood samples (Table 5). However, reductase activity in the mononuclear fraction (77% lymphocytes, 23% monocytes, <4% granulocytes [8]) was found to be stimulated to a lesser degree than the granulocyte fraction (data not shown), suggesting that granulocytes may be more sensitive to this phenomenon. This suggestion is further supported by the observation that addition of plasma-free erythrocytes to uncontaminated mononuclear leukocyte populations following density-gradient centrifugation, and subsequent erythrocyte lysis during NH_4Cl, treatment, had relatively small effects on activity of the mononuclear leukocyte enzyme (Table 6).

The data presented above describe a potential problem associated with measurement of leukocyte HMG-CoA reductase activity that may result from poor separation of leukocytes and erythrocytes and subsequent erythrocyte lysis. Furthermore, if erythrocyte lysis is the event responsible for aberrant reductase activity, then hemolysis prior to density-gradient centrifugation could also lead to increased HMG-CoA reductase activity. Because all methods for measuring leukocyte reductase activity involve separating leukocytes from erythrocytes, artificial stimulation of leukocyte reductase activity, either by hemolysis prior to separation, or by lysis of contaminating erythrocytes following density-gradient centrifugation, presents a problem whether enzyme activity is measured in isolated microsomes (8,13-18), whole cell extracts (7,10-12) or in whole cells by means of acetate conversion to cholesterol (1-6).

Erythrocyte contamination and/or lysis-induced stimulation of enzyme activity may be minimized in several ways. In obtaining blood samples, vacutainers should be avoided, because their use increases the likelihood of hemolysis. Instead, large gauge needles and syringes should be used and the blood expelled slowly into plastic containers to minimize hemolysis. Processing of blood samples through density-gradient centrifugation should be as rapid as possible to avoid storage-induced hemolysis. Hank's BSS and separation medium should be at room temperature and density-gradient centrifugation should be conducted at room temperature to permit optimal erythrocyte and leukocyte separation (20). Other methods for optimizing separation of mononuclear leukocytes from erythrocytes and granulocytes have been outlined by

TABLE 4

Leukocyte Reductase Activity is Increased by Erythrocyte Lysis

Subject	Reductase activity (pmol/min/mg)			Fold increase
	Uncontaminated mononuclear leukocytes	Total leukocytes isolated over Percoll	Total leukocytes isolated by erythrocyte lysis	
1	8.2 ± 0.01	8.3 ± 0.6	—	1.0
2	9.3 ± 0.1	10.2 ± 1.4	—	1.1
3	9.3 ± 1.0	—	146 ± 7	15.7
4	11.1 ± 1.6	—	69 ± 0.6	6.2
5	9.9 ± 0.3	—	63 ± 1.4	6.4

Leukocyte populations from 40 ml of peripheral blood were isolated by density-gradient centrifugation over Histopaque 1077 (mononuclear leukocytes) or 70% isoosmotic Percoll (total leukocytes) or by total erythrocyte lysis with NH_4Cl (total leukocytes). For leukocyte populations isolated by erythrocyte lysis, blood samples were separated into four 10-ml portions and centrifuged to remove plasma. Each pellet was then resuspended in 50 ml of 0.87% NH_4Cl and permitted to stand for 10 min at room temperature. About 50% of the erythrocytes were lysed by this treatment. Following centrifugation, each cell pellet was resuspended in 50 ml of 0.87% NH_4Cl and remaining erythrocytes were lysed during a second 10-min incubation. Further processing of the leukocyte pellets were as previously described. Data are the average of triplicate determinations of reductase activity ± SE.

TABLE 5

Granulocyte Reductase Activity is Increased by Erythrocyte Lysis

Subject	Reductase activity (pmol/min/mg)		
	Uncontaminated mononuclear leukocytes	Uncontaminated granulocytes	Contaminated granulocytes
1	8.8 ± 0.5	8.0 ± 0.5	—
2	11.5 ± 0.5	9.1 ± 0.4	—
3	7.4 ± 0.4	9.4 ± 1.4	—
4	6.9 ± 1.1	—	92
5	5.9 ± 1.0	—	76
6	9.9 ± 2.1	—	32

For subjects 1-3, mononuclear leukocytes and granulocytes were isolated from a single blood sampling by isoosmotic Percoll-gradient centrifugation, as previously described (8). For subjects 4-6, peripheral blood samples of 120 ml were separated into two fractions. Mononuclear leukocytes were isolated from one fraction (40 ml), whereas total leukocyte populations were isolated from the remaining portion (80 ml) by erythrocyte lysis (see Table 4). Following erythrocyte lysis, granulocyte populations were isolated from the total leukocyte populations by discontinuous density-gradient centrifugation. Where indicated above, the data presented are the average of triplicate determinations of leukocyte microsomal reductase activity ± SE.

TABLE 6

Variable Increase in Mononuclear Leukocyte Reductase Activity Produced by Erythrocyte Addition

Subject	Reductase activity (pmol/min/mg)		Fold increase
	No added erythrocytes	Added erythrocytes	
1	12.8 ± 0.2	38.9 ± 1.8	3.0
2	3.9 ± 0.2	6.0 ± 0.2	1.5
3	5.7 ± 0.7	5.5 ± 0.4	1.0
4	6.0 ± 0.7	10.0 ± 0.4	1.7
5	8.2 ± 0.01	8.8 ± 0.2	1.1
6	9.2 ± 0.1	11.0 ± 1.6	1.2
7	15.7 ± 2.5	14.0 ± 3.6	1.0
8	10.5 ± 0.3	16.6 ± 1.0	1.7
9	12.8 ± 0.9	12.9 ± 0.6	1.0

Peripheral blood mononuclear leukocytes were obtained by leukapheresis and subsequent density-gradient centrifugation, as previously described. Mononuclear leukocytes equivalent to that present in a 40-ml blood sample were mixed with 40 ml Hank's BSS or 40 ml Hank's BSS containing 80 μl of a plasma-free erythrocyte fraction (an amount sufficient to provide a homogeneously red leukocyte pellet). Following centrifugation, the cell pellets were washed in Hank's BSS and the erythrocytes lysed in 0.87% NH_4Cl. Further processing of the leukocyte pellets was as previously described. Data are the averages of triplicate determinations of reductase activity ± SE. For subjects 8 and 9, lysed erythrocytes were used in place of whole erythrocytes.

Boyun (20). When isolating total leukocyte populations by Percoll density-gradient centrifugation, the possibility of significant erythrocyte contamination is greater than during mononuclear leukocyte isolation, however, because some erythrocyte aggregates remain suspended in the Percoll layer following centrifugation. Care must be taken not to include these aggregates in the leukocyte layer.

When these precautions are followed, reproducible determination of leukocyte reductase activity may be achieved (8,13–17). However, any undue time required for processing blood samples or suspected hemolysis or visual erythrocyte contamination of the leukocyte fraction following density-gradient centrifugation should be noted and the validity of subsequent reductase activity determinations questioned.

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REFERENCES

1. McNamara, D.J., Davidson, N.O., and Fernandez, S. (1980) *J. Lipid Res. 21,* 65–71.
2. Fogelman, A.M., Seager, J., Edwards, P.A., Hokom, M., and Popjak, G. (1977) *Biochem. Biophys. Res. Commun. 76,* 167–173.
3. Krone, W., Betteridge, D.J., and Galton, D.J. (1979) *Eur. J. Clin. Invest. 9,* 405–410.
4. Ho, Y.K., Smith, R.G., Brown, M.S., and Goldstein, J.L. (1978) *Blood 52,* 1099–1114.
5. Yachnin, S., Larson, R.A., and West, E.J. (1983) *Brit. J. Hematol. 54,* 459–466.
6. Chen, H.W., Heiniger, H.J., and Kandutsch, A.A. (1977) *Exp. Cell. Res. 109,* 253–262.
7. Fogelman, A.M., Edmond, J., Seager, J., and Popjak, G. (1980) *J. Biol. Chem. 25,* 2045–2055.
8. Harwood, H.J., Jr., Schneider, M., and Stacpoole, P.W. (1984) *J. Lipid Res. 25,* 967–978.
9. Shechter, I., Fogelman, A.M., and Popjak, G. (1980) *J. Lipid Res. 21,* 277–283.
10. Knight, B.L., Patel, D.D., and Soutar, A.K. (1983) *Biochem. J. 210,* 523–532.
11. Tabacik, C., Astruc, M., LaPorte, M., Descomps, B., Crastes de Paulet, A., and Serrou, B. (1981) *Biomedicine 34,* 128–132.
12. Young, N.L. and Berger, B. (1981) *Methods Enzymol. 71:*489–509.
13. Harwood, H.J. Jr., Schneider, M., and Stacpoole, P.W. (1984) *Biochim. Biophys. Acta 805,* 245–251.
14. Harwood, H.J. Jr., Greene, Y.J., and Stacpoole, P.W. (1986) *J. Biol. Chem. 261,* 7127–7135.
15. Harwood, H.J. Jr., Alvarez, I.M., Bridge, D.M., Greene, Y.J., and Stacpoole, P.W. (1986) *Fed. Proc. 45,* 1547.
16. Harwood, H.J. Jr., Alvarez, I.M., Greene, Y.J., Noyes, W.D., and Stacpole, P.W. (1986) *Clin. Res. 34,* 564A.
17. Harwood, H.J. Jr., Bridge, D.M., and Stacpoole, P.W. (1987) *J. Clin. Invest. 79,* 1125–1132.
18. Harwood, H.J. Jr., Alvarez, I.M., Greene, Y.J., Ness, G.C., and Stacpoole, P.W. (1987) *J. Lipid Res. 28,* 292–304.
19. Bradford, M.M. (1976) *Anal. Biochem. 72,* 248–254.
20. Boyum, A. (1968) *Scand. J. Clin. Lab Invest. Suppl. 97.21,* 77–89.
21. Stacpoole, P.W., Bridge, D.M., Alvarez, I.M., Goldberg, R.B., and Harwood, H.J. Jr. (1987) *J. Clin. Invest. 80,* 1401–1408.

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ERRATUM

"Effect of Essential Fatty Acid Deficiency on the Size and Distribution of Rat Plasma HDL" by K. E. Lowe, S. Pelkey, M. A. Williams, and A. V. Nichols, *Lipids 23,* 106–114, 1988. Reference 37 should read:

37. Ney, D.M., Ziboh, V.A., and Schneeman, B.O. (1987) *J. Nutr. 117,* 666–672.

Boyun (20). When isolating total leukocyte populations by Percoll density-gradient centrifugation, the possibility of significant erythrocyte contamination is greater than during mononuclear leukocyte isolation, however, because some erythrocyte aggregates remain suspended in the Percoll layer following centrifugation. Care must be taken not to include these aggregates in the leukocyte layer.

When these precautions are followed, reproducible determination of leukocyte reductase activity may be achieved (8,13–17). However, any undue time required for processing blood samples or suspected hemolysis or visual erythrocyte contamination of the leukocyte fraction following density-gradient centrifugation should be noted and the validity of subsequent reductase activity determinations questioned.

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REFERENCES

1. McNamara, D.J., Davidson, N.O., and Fernandez, S. (1980) *J. Lipid Res. 21,* 65–71.
2. Fogelman, A.M., Seager, J., Edwards, P.A., Hokom, M., and Popjak, G. (1977) *Biochem. Biophys. Res. Commun. 76,* 167–173.
3. Krone, W., Betteridge, D.J., and Galton, D.J. (1979) *Eur. J. Clin. Invest. 9,* 405–410.
4. Ho, Y.K., Smith, R.G., Brown, M.S., and Goldstein, J.L. (1978) *Blood 52,* 1099–1114.
5. Yachnin, S., Larson, R.A., and West, E.J. (1983) *Brit. J. Hematol. 54,* 459–466.
6. Chen, H.W., Heiniger, H.J., and Kandutsch, A.A. (1977) *Exp. Cell. Res. 109,* 253–262.
7. Fogelman, A.M., Edmond, J., Seager, J., and Popjak, G. (1980) *J. Biol. Chem. 25,* 2045–2055.
8. Harwood, H.J., Jr., Schneider, M., and Stacpoole, P.W. (1984) *J. Lipid Res. 25,* 967–978.
9. Shechter, I., Fogelman, A.M., and Popjak, G. (1980) *J. Lipid Res. 21,* 277–283.
10. Knight, B.L., Patel, D.D., and Soutar, A.K. (1983) *Biochem. J. 210,* 523–532.
11. Tabacik, C., Astruc, M., LaPorte, M., Descomps, B., Crastes de Paulet, A., and Serrou, B. (1981) *Biomedicine 34,* 128–132.
12. Young, N.L. and Berger, B. (1981) *Methods Enzymol. 71:*489–509.
13. Harwood, H.J. Jr., Schneider, M., and Stacpoole, P.W. (1984) *Biochim. Biophys. Acta 805,* 245–251.
14. Harwood, H.J. Jr., Greene, Y.J., and Stacpoole, P.W. (1986) *J. Biol. Chem. 261,* 7127–7135.
15. Harwood, H.J. Jr., Alvarez, I.M., Bridge, D.M., Greene, Y.J., and Stacpoole, P.W. (1986) *Fed. Proc. 45,* 1547.
16. Harwood, H.J. Jr., Alvarez, I.M., Greene, Y.J., Noyes, W.D., and Stacpole, P.W. (1986) *Clin. Res. 34,* 564A.
17. Harwood, H.J. Jr., Bridge, D.M., and Stacpoole, P.W. (1987) *J. Clin. Invest. 79,* 1125–1132.
18. Harwood, H.J. Jr., Alvarez, I.M., Greene, Y.J., Ness, G.C., and Stacpoole, P.W. (1987) *J. Lipid Res. 28,* 292–304.
19. Bradford, M.M. (1976) *Anal. Biochem. 72,* 248–254.
20. Boyum, A. (1968) *Scand. J. Clin. Lab Invest. Suppl. 97.21,* 77–89.
21. Stacpoole, P.W., Bridge, D.M., Alvarez, I.M., Goldberg, R.B., and Harwood, H.J. Jr. (1987) *J. Clin. Invest. 80,* 1401–1408.

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ERRATUM

"Effect of Essential Fatty Acid Deficiency on the Size and Distribution of Rat Plasma HDL" by K. E. Lowe, S. Pelkey, M. A. Williams, and A. V. Nichols, *Lipids 23,* 106–114, 1988. Reference 37 should read:

37. Ney, D.M., Ziboh, V.A., and Schneeman, B.O. (1987) *J. Nutr. 117,* 666–672.